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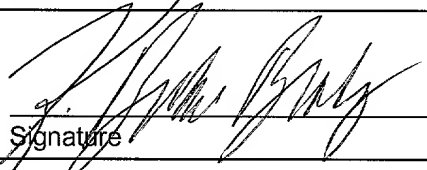


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### UTILITY PATENT APPLICATION TRANSMITTAL UNDER 37 CFR §1.53(b)

Attorney Docket Number	50004/003003
Applicant	Roy A. Gravel, Rima Rozen, Daniel Leclerc, Aaron Wilson, and David Rosenblatt
Title	HUMAN METHIONINE SYNTHASE REDUCTASE: CLONING, AND METHODS FOR EVALUATING RISK OF NEURAL TUBE DEFECTS, CARDIOVASCULAR DISEASE, AND CANCER
<b>PRIORITY INFORMATION:</b>	
This application is a continuation-in-part of and claims priority from United States patent application 09/232,028, filed January 15, 1999, which claims priority from U.S. Provisional Application No. 60/071,622, filed January 16, 1998.	
<b>APPLICATION ELEMENTS:</b>	
Cover sheet	1 pages
Specification	61 pages
Claims	7 pages
Abstract	1 pages
Drawing	10 pages
Combined Declaration and POA, which is: <input checked="" type="checkbox"/> Unsigned; <input type="checkbox"/> Newly signed for this application; <input type="checkbox"/> A copy from prior application [**SERIAL NUMBER**] and the entire disclosure of the prior application is considered as being part of the disclosure of this new application and is hereby incorporated by reference therein.	4 pages
Statement Deleting Inventors	0 pages
Sequence Statement	0 pages
Sequence Listing on Paper	0 pages
Sequence Listing on Diskette	0 pages

660780 "44T2E60"

Small Entity Statement, which is: <input type="checkbox"/> Unsigned; <input type="checkbox"/> Newly signed for this application; <input checked="" type="checkbox"/> A copy from prior application 09/232,028 and such small entity status is still proper and desired.	1 page
Preliminary Amendment	0 pages
IDS	0 pages
Form PTO 1449	0 pages
Cited References	0 pages
Recordation Form Cover Sheet and Assignment	0 pages
Assignee's Statement	0 pages
English Translation	0 pages
Certified Copy of Priority Document	0 pages
Return Receipt Postcard	1
<b>FILING FEES:</b>	
Basic Filing Fee: \$380	\$ 380.00
Excess Claims Fee: 35 - 20 x \$9	\$ 135.00
Excess Independent Claims Fee: 12 - 3 x \$39	\$ 351.00
Multiple Dependent Claims Fee: \$130	\$ 130.00
Total Fees:	\$ 996.00
<input checked="" type="checkbox"/> Enclosed is a check for \$996.00 to cover the total fees. <input type="checkbox"/> Charge [**AMOUNT**] to Deposit Account No. 03-2095 to cover the total fees. <input type="checkbox"/> The filing fee is not being paid at this time. <input checked="" type="checkbox"/> Please apply any other charges, or any credits, to Deposit Account No. 03-2095.	
<b>CORRESPONDENCE ADDRESS:</b>	
Kristina Bieker-Brady, Ph.D. Reg. No. 39,109 Clark & Elbing LLP 176 Federal Street Boston, MA 02110 <div style="text-align: right;">             Telephone: 617-428-0200              Facsimile: 617-428-7045           </div>	
 Signature	August 10, 1999 Date

Applicant or Patentee : Roy A. Gravel et al.  
 Serial or Patent No. : 09/232,028  
 Filed or Issued : January 15, 1999  
 Title : HUMAN METHIONINE SYNTHASE REDUCTASE: CLONING, AND METHODS FOR  
 EVALUATING RISK OF NEURAL TUBE DEFECTS, CARDIOVASCULAR DISEASE, AND CANCER

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
 (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: McGill University  
 Address of Organization: 845 Sherbrooke Street West, Montreal Quebec H3A 2T5 Canada  
 Type of Organization:

- ☒ University or Other Institution of Higher Education  
☐ Tax Exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))  
☐ Nonprofit Scientific or Educational under Statute of State of the United States of America  
 Name of State:  
 Citation of Statute:  
☐ Would Qualify as Tax Exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)) If Located in the United States of America  
☐ Would Qualify as Nonprofit Scientific or Educational under Statute of State of the United States of America If Located in the United States of America  
 Name of State:  
 Citation of Statute:

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled HUMAN METHIONINE SYNTHASE REDUCTASE: CLONING, AND METHODS FOR EVALUATING RISK OF NEURAL TUBE DEFECTS, CARDIOVASCULAR DISEASE, AND CANCER by inventors Roy A. Gravel, Rima Rozen, Daniel Lederc, Aaron Wilson, and David Rosenblatt described in

- ☐ the specification filed herewith.  
☒ application serial no. 09/232,028, filed January 15, 1999.  
☐ patent no. **[\*\*PATENT NUMBER\*\*]**, issued **[\*\*ISSUE DATE\*\*]**.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

\*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name:  
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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

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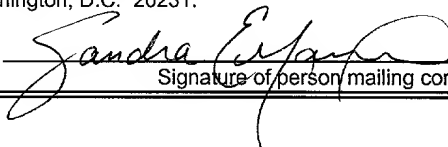
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APPLICATION

FOR

UNITED STATES LETTERS PATENT

APPLICANT : ROY A. GRAVEL, RIMA ROZEN, DANIEL LECLERC,  
AARON WILSON, AND DAVID ROSENBLATT

TITLE : HUMAN METHIONINE SYNTHASE REDUCTASE:  
CLONING, AND METHODS FOR EVALUATING RISK OF  
NEURAL TUBE DEFECTS, CARDIOVASCULAR DISEASE,  
AND CANCER

HUMAN METHIONINE SYNTHASE REDUCTASE: CLONING, AND  
METHODS FOR EVALUATING RISK OF NEURAL TUBE DEFECTS,  
CARDIOVASCULAR DISEASE, AND CANCER

Cross-Reference to Related Applications

This application is a continuation-in-part of U.S. application serial no. 09/232,028 entitled "Methods for Evaluating Risk of Neural Tube Defects, Cardiovascular Disease, and Cancer" filed by Gravel et al. on January 15, 1999, which claims priority from U.S. Provisional Application No. 60/071,622, filed January 16, 1998.

Field of the Invention

This invention relates to the diagnosis and treatment of patients at risk for disorders associated with altered methionine synthase activity.

Background of the Invention

Methionine is an essential amino acid in mammals that is required for protein synthesis. Methionine also plays a central role in metabolic reactions involving transfer of single-carbon moieties: in its activated form, S-adenosylmethionine, methionine is the methyl donor in hundreds of biological transmethylation reactions. Moreover, methionine is the propylamine donor in polyamine synthesis. The ultimate product resulting from the demethylation of methionine is homocysteine, the remethylation of which is catalyzed by a cobalamin-dependent enzyme, methionine synthase (5-methyltetrahydrofolate:homocysteine methyltransferase, EC 2.1.1.13).

The enzyme-bound cobalamin cofactor of methionine synthase plays an essential role in the methyl transfer reaction by acting as an intermediate methyl carrier between methyltetrahydrofolate and homocysteine. The upper portion of Fig. 1 illustrates the transfer of the methyl group of methyltetrahydrofolate (CH<sub>3</sub>-THF) to homocysteine via methionine synthase-methylcobalamin [MetSyn-CH<sub>3</sub>-Co(III)] as an intermediate methyl carrier. Cleavage of the methyl-cobalt bond of the methylcob(III)alamin intermediate occurs heterolytically so as to leave the cobalamin in the highly reactive cob(I)alamin oxidation state. The occasional oxidation of the enzyme-cobalamin to the cob(II)alamin state [MetSyn-Co(II)] renders the enzyme inactive.

Severe deficiency of methionine synthase activity leads to megaloblastic anemia, developmental delay, hyperhomocysteinemia, and hypomethioninemia. Moreover, elevated plasma homocysteine is a risk factor in cardiovascular disease and neural tube defects (Rozen, *Clin. Invest. Med.* 19:171-178, 1996).

Two forms of methionine synthase deficiency are known (Watkins et al., *Am. J. Med. Genet.* 34:427-434, 1989; Gulati et al., *J. Biol. Chem.* 272:19171-19175, 1997). The first is a primary defect of the amino acid sequence of the methionine synthase enzyme. We recently cloned cDNAs encoding human methionine synthase and showed that patients from the *cblG* complementation group of folate/cobalamin metabolism have mutations in the methionine synthase gene. A second class of patients, belonging to a distinct complementation group, *cblE*, is also deficient in methionine synthase enzymatic activity. The genetic basis of this deficiency has not been determined.

An analogous methylcobalamin-dependent methionine synthase has been well characterized in *E. coli* and the structures comprising its C-terminal half

have been elucidated by X-ray crystallography. The reductive activation system required for its maintenance is a two-component flavoprotein system consisting of flavodoxin (a small FMN-containing electron transfer protein), and NADPH-ferredoxin (flavodoxin) oxidoreductase, a member of a family of electron transferases termed the "FNR family." However, flavodoxins are not found in mammalian cells.

It would be desirable to identify the enzyme that catalyzes the reductive activation of methionine synthase, i.e., the methionine synthase reductase. Knowledge of the reductase wild-type nucleotide and amino acid sequences would allow the identification of mutations and polymorphisms associated with diseases involving methionine metabolism. Moreover, an understanding of the reductase structure and function will facilitate the identification of compounds that modulate its activity. Such compounds will be useful in treating and preventing disease and developmental defects.

#### Brief Description of the Drawings

Fig. 1 is a diagram showing the enzymatic reaction that is catalyzed by methionine synthase, and the reductive reactivation of methionine synthase.

Fig. 2 is a diagram showing the overlapping clones and PCR fragments used to clone and sequence human methionine synthase reductase.

Fig. 3 is a diagram showing the nucleotide and deduced amino acid sequence of human methionine synthase reductase.

Fig. 4 is a diagram showing an amino acid sequence comparison among human methionine synthase reductase (HsMTRR; SEQ ID NO: 21), *C. elegans* putative methionine synthase reductase (CeMTRR; SEQ ID NO: 22) and human

cytochrome P450 reductase (HsCPR; SEQ ID NO: 23).

Figs. 5A and 5B are representations of Northern blots showing an analysis of methionine synthase reductase expression in human tissues.

Fig. 6 is a diagram summarizing the FISH mapping of the methionine synthase reductase gene to human chromosome 5p15.2-p15.3.

Figs. 7A and 7B are representations of gels showing a mutation analysis of *cblE* patient cell lines.

Fig. 7C is a diagram showing a sequence comparison of the NADPH binding region of FNR family members (SEQ ID NOs: 25-40)

Fig. 8A is a representation of two autoradiograms showing the A to G polymorphism at MTRR coding position 66.

Fig. 8B is a representation of a gel showing a restriction digest assay for distinguishing between the adenine 66 and guanine 66 alleles.

#### Summary of the Invention

We have cloned the gene encoding human methionine synthase reductase. This enzyme maintains methionine synthase in its reduced, activated state, and hence is an essential component of the methionine synthetic pathway. Deficiency of methionine synthase reductase results in hyperhomocysteinemia, a condition that has been implicated in cardiovascular disease and neural tube defects. The presence of mutations in the methionine synthase reductase gene that decrease methionine synthase reductase enzymatic activity are likely to be associated with altered risk for cardiovascular disease, neural tube defects, and cancer. The invention features methods for risk detection and treatment of patients with hyperhomocysteinemia, cardiovascular disease, neural tube defects, and



cancer. The invention also features compounds and kits which may be used to practice the methods of the invention, methods and compounds for treating or preventing these conditions and methods of identifying therapeutics for the treatment or prevention of these conditions.

5           In a first aspect, the invention features substantially pure nucleic acid encoding a mammalian methionine synthase reductase polypeptide. In various embodiments, the nucleic acid may encode a human polypeptide, and the nucleic acid may be DNA, particularly genomic DNA or cDNA. In another embodiment, the nucleic acid has the sequence of SEQ ID NO: 1 or SEQ ID NO: 41, or  
10   degenerate variants thereof, and the nucleic acid encodes the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 42. In yet another embodiment, the nucleic acid is operably linked to regulatory sequences for expression of methionine synthase reductase. The regulatory sequences comprise a promoter, and the promoter may be inducible.

15           In a second, related aspect, the invention features a substantially pure nucleic acid that hybridizes at high stringency to the nucleic acid of SEQ ID NO: 1 or SEQ ID NO: 41. In a preferred embodiment, the nucleic acid is a naturally occurring variant of the mammalian methionine synthase reductase gene. In another embodiment, the nucleic acid has a sequence complementary to at least  
20   50% of at least 60 nucleotides of the nucleic acid encoding the methionine synthase reductase polypeptide, and the sequence is sufficient to allow nucleic acid hybridization under high stringency conditions. In further embodiments, the nucleic acid may be a probe or an antisense nucleic acid, and the sequence may be complementary to at least 90% of at least 18 nucleotides of the nucleic acid  
25   encoding the methionine synthase reductase polypeptide.

In a third aspect, the invention features a nucleic acid encoding a mutant or polymorphic mammalian methionine synthase reductase polypeptide. In one embodiment, the nucleic acid may be from a human. In another embodiment, the mutation is a deletion mutation, for example, a deletion of 4 bases starting from  
 5 base 1675 (bases 1675-1678) of SEQ ID NO:1 (SEQ ID NO: 47), or a deletion of 3 bases starting from base 1726 (bases 1726-1728) of SEQ ID NO:1 (SEQ ID NO: 45). In still another embodiment the polymorphism is a nucleotide transition from G to A at nucleotide position 66 (SEQ ID NO: 41), or from G to A at nucleotide position 110 (SEQ ID NO: 43). Other naturally-occurring variants associated with  
 10 altered risk for hyperhomocysteinemia are also a feature of this aspect of the invention.

In a fourth, related aspect, the invention features a cell containing the nucleic acid of the third aspect of the invention. In various embodiments, the cell may be a prokaryotic cell, a eukaryotic cell, a yeast cell, or a mammalian cell.

15 In a fifth, related aspect, the invention features a non-human transgenic animal containing the nucleic acid of the third aspect of the invention. In one embodiment, the nucleic acid contains a mutation associated with hyperhomocysteinemia.

In a sixth, related aspect, the invention features a non-human animal  
 20 wherein one or both genetic alleles encoding a methionine synthase reductase polypeptide are mutated. In one embodiment of this sixth aspect, one or both genetic alleles encoding a methionine synthase reductase polypeptide are disrupted, deleted, or otherwise rendered nonfunctional. In further embodiments of the fifth and sixth aspects, the animal may be a rodent (e.g., a mouse), or a  
 25 nematode (e.g., *C. elegans*).

In a seventh, related aspect, the invention features a cell from the animal of the fifth and sixth aspects.

In an eighth aspect, the invention features a substantially pure mammalian methionine synthase reductase polypeptide. In various embodiments, the polypeptide may be recombinant, or may be a human polypeptide, or may be the polypeptide set forth in SEQ ID NO: 2 or SEQ ID NO: 42.

In a ninth, related aspect, the invention features a polypeptide having conservative amino acid substitutions relative to SEQ ID NO: 2 or SEQ ID NO: 42, and having methionine synthase reductase biological activity.

In a tenth, related aspect, the invention features a mutant or polymorphic polypeptide which has less methionine synthase reductase biological activity than the polypeptide of SEQ ID NO: 2. In preferred embodiments, the polypeptide has a frameshift resulting in a premature stop codon (e.g., SEQ ID NO: 48), or a deletion mutation, such as a deletion of Leu576 (SEQ ID NO: 46). In other preferred embodiments, the polypeptide may have an amino acid substitution, such as isoleucine instead of methionine at amino acid position 22 (SEQ ID NO: 42), or tyrosine instead of cysteine at amino acid position 37 (SEQ ID NO: 44).

In an eleventh, related aspect, the invention features a mutant or polymorphic polypeptide which has higher methionine synthase reductase biological activity than the polypeptide set forth in SEQ ID NO: 2.

In a twelfth aspect, the invention features an antibody that specifically binds a methionine synthase reductase polypeptide. In one embodiment, the polypeptide is a mutant or polymorphic polypeptide.

In a thirteenth, related aspect, the invention features a method of generating an antibody that specifically binds a methionine synthase reductase

polypeptide. The method comprises administering a methionine synthase reductase polypeptide, or fragment thereof, to an animal capable of generating an immune response, and isolating the antibody from the animal. Preferred antibodies specifically bind mutant methionine synthase reductase polypeptides.

5 In a fourteenth, related aspect, the invention features a method of detecting the presence of a methionine synthase reductase polypeptide. The method comprises contacting a sample with the antibody that specifically binds a methionine synthase reductase polypeptide and assaying for binding of the antibody to the polypeptide.

10 In a fifteenth aspect, the invention features a method for detecting sequence variants for methionine synthase reductase in a mammal. The method comprises analyzing the nucleic acid of a test subject to determine whether the test subject contains a mutation or polymorphism in a methionine synthase reductase gene. The presence of the mutation or polymorphism is an indication that the  
15 animal has an increased or decreased likelihood of developing hyperhomocysteinemia, cardiovascular disease, neural tube defects, or cancer.

In one embodiment of the fifteenth aspect, primers used for detecting a mutation are selected from: 5'-CTCCTGCTCGAACATCTTCCTAAA (SEQ ID NO: 3); 5'-AATAGATAAT CCCTATCCTTATGCC (SEQ ID NO: 4); 5'-  
20 CCCTGGCTCCTAAGATATCCATC (SEQ ID NO: 5); 5'-CGAACAACAAA TTCTTTCCACTTACC (SEQ ID NO: 6); 5'-CAAGGTTGGTGGAA GTCGCGTTG (SEQ ID NO: 7); 5'-ATGCCTTGAAGTGAT GAGGAGGTTT (SEQ ID NO: 8); 5'-TTCCTACAACATAGAGAGAACTC (SEQ ID NO: 9); 5'-  
25 TTGCACAAGGGCATCATGTACATC (SEQ ID NO: 10); 5'-AAACCTCC TCATCACTTCAAGGCAT (SEQ ID NO: 11); 5'-CTTGCACACGAATATG

GTCTGGG (SEQ ID NO: 12); 5'-TGGCATCACCTGCATCCTTGAGG (SEQ ID NO: 13); 5'-GATGTACCTGTAAATATTCTGGGGG (SEQ ID NO: 14); 5'-AATCCACGGCTCAA CCACAAGTTC (SEQ ID NO: 15); 5'-CTCGAAATT AACCTCACTAAAGGG (SEQ ID NO: 16); 5'-AACCCATACCGCAG GTGAGCAA (SEQ ID NO: 17); 5'-TTAGTACTTTCAGTCAAAAAA GCTTAAT (SEQ ID NO: 18); 5'-ATAAACGACTTCAAGA GCTTGGAGC (SEQ ID NO: 19); or 5'-AGGTTTGGCACTAGTAAAGCTGACT (SEQ ID NO: 20).

In another embodiment of the fifteenth aspect of the invention, the method further comprises the step of using nucleic acid primers specific for the methionine synthase reductase gene. The primers are used for DNA amplification by the polymerase chain reaction. In yet another embodiment, the step further comprises the step of sequencing nucleic acid encoding methionine synthase reductase from the test subject. In still other embodiments, the analyzing includes single strand conformational polymorphism (SSCP) analysis, or the method is carried out by restriction fragment length (RFLP) polymorphism analysis. In further embodiments, the method is for the diagnosis of an altered risk for cardiovascular disease, neural tube defects, or cancer, such as colon cancer.

In a sixteenth aspect, the invention features a kit for the analysis of mammalian methionine synthase reductase nucleic acid. The kit comprises nucleic acid probes for analyzing the nucleic acid of a mammal, and the analyzing is sufficient to determine whether the mammal contains a mutation in the methionine synthase reductase nucleic acid. In a preferred embodiment the nucleic acid probes allow detection of mutations associated with hyperhomocysteinemia.

In a seventeenth aspect, the invention features a kit for the analysis of

mammalian methionine synthase reductase polypeptides. The kit comprises antibodies for analyzing the methionine synthase reductase polypeptide of a mammal, and the analyzing is sufficient to determine whether the mammal contains a mutation in the methionine synthase reductase nucleic acid.

5 In an eighteenth aspect, the invention features a method of treating or preventing cancer, cardiovascular disease, or neural tube defects. The method comprises inhibiting methionine synthase reductase biological activity. In one embodiment, the mammal is pregnant. In other embodiments, the method comprises administering a therapeutically effective dose of a methionine synthase  
10 reductase inhibitor to a mammal. The inhibitor may be a methionine synthase reductase anti-sense nucleic acid, a peptide comprising a portion of a mammalian methionine synthase reductase polypeptide, or a small molecule.

In a nineteenth aspect, the invention features a method of treating or preventing cardiovascular disease. The method comprises administering to the  
15 subject a therapeutically effective dose of a metabolite or cofactor selected from the group: folate, cobalamin, S-adenosyl methionine, betaine, or methionine.

In a twentieth aspect, the invention features a method of preventing neural tube defects, cancer, or cardiovascular disease. The method comprises: a) detecting an increased risk of neural tube defects, cancer, or cardiovascular  
20 disease, wherein the detecting is performed by analyzing methionine synthase reductase nucleic acid from one or more test subjects selected from: a mammal; a potential parent, either male or female; a pregnant mammal; or a developing embryo or fetus, wherein the analyzing is done by the method of the fifteenth aspect of the invention; and b) exposing the mammal, potential parent, pregnant  
25 mammal, and/or developing embryo or fetus to a therapeutically effective dose of

a metabolite or cofactor selected from the group: cobalamin; S-adenosyl methionine; betaine; or methionine, wherein the exposing is via the administration of the dose to the mammal, the potential parent, the pregnant mammal, and/or the developing embryo or fetus.

5           In a preferred embodiment of the eighteenth and twentieth aspects of the invention, the subject has been diagnosed as having a mutation or polymorphism in methionine synthase reductase.

10           In a twenty-first aspect, the invention features a method of screening for a compound that modulates methionine synthase reductase biological activity. The method comprises the steps of: a) contacting a sample containing wild-type, mutated, or polymorphic methionine synthase reductase with the compound, and b) assaying for methionine synthase reductase enzymatic activity, wherein increased enzymatic activity indicates an inducer of methionine synthase reductase biological activity, and decreased enzymatic activity indicates an inhibitor of  
15           methionine synthase reductase biological activity.

20           In a twenty-second aspect, the invention features a method for screening for a compound that modulates methionine synthase reductase biological activity. The method comprises the steps of: a) contacting a sample with the compound, and b) assaying for methionine synthase reductase expression, wherein increased  
25           expression indicates an inducer of methionine synthase reductase biological activity, and decreased expression indicates an inhibitor of methionine synthase reductase biological activity. The sample is selected from: purified or partially purified methionine synthase reductase, a cell lysate, a cell, a nematode, or a mammal. In preferred embodiments, the sample may be the animal or cell described by the fifth and sixth aspects of the invention. In other preferred

embodiments, the screening may be for compounds useful for the treatment or prevention of cardiovascular disease or cancer, or for the prevention of neural tube defects.

In a twenty-third aspect, the invention features a method for detecting an increased risk of developing a neural tube defect in a mammalian embryo or fetus. The method includes detecting the presence of a polymorphic methionine synthase reductase (MTRR) in a test subject, wherein the polymorphic MTRR contains a methionine instead of an isoleucine at amino acid position 22, wherein the test subject is a future parent of the embryo or fetus, and wherein detection of a homozygous MTRR polymorphism in the future parent, embryo, or fetus, or detection of either a homozygous or heterozygous MTRR polymorphism in both future parents, indicates an increased risk of developing a neural tube defect in the embryo or fetus.

In various embodiments of the twenty-third aspect of the invention, the polymorphic MTRR may be detected by analyzing nucleic acid from the test subject. The nucleic acid may be genomic DNA or cDNA. The nucleic acid may contain a G instead of an A at the third position of the twenty-second codon (nucleotide position 66, relative to the first nucleotide of the start codon) of MTRR.

In another embodiment of the twenty-third aspect of the invention, the method may further include: a) PCR-amplifying a segment of MTRR nucleic acid using primers MSG108S (SEQ ID NO: 49) and AD292 (SEQ ID NO: 50), and b) digesting the product of the PCR amplification reaction with the restriction enzyme *Nde* I, wherein a PCR product that is digested by *Nde* I indicates an increased risk of developing a neural tube defect in a mammalian embryo or fetus.



In still other embodiments of the twenty-third aspect of the invention, the polymorphic MTRR may be detected by analyzing MTRR polypeptide from the test subject, and the test subject may be a future female parent of the embryo or fetus, or the

5 test subject may be the embryo or fetus itself.

In yet further embodiments of the twenty-third aspect of the invention, the method may further include detecting the presence of a polymorphic methylenetetrahydrofolate reductase (MTHFR) in a test subject, the polymorphic MTHFR having a T instead of a C at a nucleotide position equivalent to position  
10 677 of SEQ ID NO: 51, wherein detection of the polymorphic MTHFR indicates an increased risk of developing a neural tube defect in the embryo or fetus. The polymorphic MTHFR may be detected by analyzing nucleic acid or polypeptide from the test subject.

In still another embodiment of the twenty-third aspect of the invention,  
15 the method may further include measuring the level of cobalamin in the test subject, wherein a low cobalamin level indicates an increased risk of developing a neural tube defect in the embryo or fetus.

By "methionine synthase reductase," "methionine synthase reductase protein," or "methionine synthase reductase polypeptide" is meant a polypeptide,  
20 or fragment thereof, which has at least 43% amino acid sequence identity, or at least 53% sequence similarity, preferably at least 47% identity (or at least 57% similarity), more preferably at least 55% identity (or at least 65% similarity), yet more preferably at least 65% sequence identity (or at least 75% similarity), still more preferably at least 75% sequence identity (or at least 85% similarity) and  
25 most preferably at least 85% sequence identity (or at least 95% similarity) to the

human methionine synthase reductase polypeptide of SEQ ID NO: 2 (see Fig. 4), over the length of the polypeptide or fragment thereof, or over the length of the human methionine synthase reductase polypeptide of SEQ ID NO: 2, whichever is shorter in length. It is understood that polypeptide products from splice variants of methionine synthase reductase gene sequences are also included in this definition. Preferably, the methionine synthase reductase protein is encoded by nucleic acid having a sequence which hybridizes to a nucleic acid sequence present in SEQ ID NO: 1 (human methionine synthase reductase cDNA) under stringent conditions. Even more preferably the encoded polypeptide also has methionine synthase reductase biological activity, or is a mutant or polymorphic form of methionine synthase reductase that is associated with an increased risk of disease.

By “methionine synthase reductase nucleic acid” or “methionine synthase reductase gene” is meant a nucleic acid, such as genomic DNA, cDNA, or mRNA, that encodes methionine synthase reductase, a methionine synthase reductase protein, methionine synthase reductase polypeptide, or portion thereof, as defined above.

By “mutant methionine synthase reductase,” “methionine synthase reductase mutation(s),” “mutations in methionine synthase reductase,” “polymorphic methionine synthase reductase,” “methionine synthase reductase polymorphism(s),” “polymorphisms in methionine synthase reductase,” is meant a methionine synthase reductase (MTTR) polypeptide or nucleic acid having a sequence that confers an increased risk of a disease phenotype or enhanced protection against a disease in at least some genetic and/or environmental backgrounds. An example of a disease-associated methionine synthase reductase polymorphism is the 22M polymorphism (SEQ ID NO: 2), which is associated

with an increased risk for neural tube defects.

Any given methionine synthase reductase polymorphism may be associated with an increased risk for some diseases and a decreased risk for other diseases. Increased or decreased disease risks associated with specific

5 methionine synthase reductase mutations and polymorphisms are determined by methods known to those skilled in the art.

Such mutations may be naturally occurring, or artificially induced.

They may be, without limitation, transition, transversion, insertion, deletion, frameshift, or missense mutations. A mutant methionine synthase reductase

10 protein may have one or more mutations, and such mutations may affect different aspects of methionine synthase reductase biological activity (protein function), to various degrees. Alternatively, a methionine synthase reductase mutation may indirectly affect methionine synthase reductase biological activity by influencing, for example, the transcriptional activity of a gene encoding methionine synthase  
15 reductase, or the stability of methionine synthase reductase mRNA. For example, a mutant methionine synthase reductase gene may be a gene that expresses a mutant methionine synthase reductase protein or may be a gene which alters the level of methionine synthase reductase protein in a manner sufficient to confer a disease phenotype in at least some genetic and/or environmental backgrounds.

20 The presence of polymorphic or mutant methionine synthase reductase may be determined by detecting polymorphic or mutant methionine synthase reductase nucleic acid or polypeptide, using methods that are known in the art.

By “biologically active” methionine synthase reductase is meant a methionine synthase reductase protein or methionine synthase reductase gene that  
25 provides at least one biological function equivalent to that of the wild-type

methionine synthase reductase polypeptide or the methionine synthase reductase gene. Biological activity of a methionine synthase reductase polypeptide includes, but is not limited to, the ability to catalyze the reductive methylation of enzymatically inactive methionine synthase-cob(II)alamin to generate

- 5 enzymatically active methionine synthase-cob(III)alamin-CH<sub>3</sub>. Preferably, a biologically active methionine synthase reductase will display activity equivalent to at least 20-30% of wild-type activity, more preferably, at least 35-50% of wild-type activity, still more preferably, 55-75% of wild-type activity, and most preferably, a biologically active methionine synthase reductase will display at least 80-90% of wild-type activity. A biologically active methionine synthase reductase also may display more than 100% of wild-type activity. Preferably, the biological activity of the wild-type methionine synthase reductase is determined using the methionine synthase reductase nucleic acid of SEQ ID NO: 1 or SEQ ID NO: 41 or methionine synthase reductase polypeptide of SEQ ID NO: 2 or SEQ ID NO: 42. The degree of methionine synthase reductase biological activity may be intrinsic to the methionine synthase reductase polypeptide itself, or may be modulated by increasing or decreasing the number of methionine synthase reductase polypeptide molecules present intracellularly.

By "high stringency conditions" is meant hybridization in 2X SSC at 40°C with a DNA probe length of at least 40 nucleotides. For other definitions of high stringency conditions, see Ausubel et al., Current Protocols in Molecular Biology, pp. 6.3.1-6.3.6, John Wiley & Sons, New York, NY, 1998, hereby incorporated by reference.

By "analyzing" or "analysis" is meant subjecting a methionine synthase reductase nucleic acid or methionine synthase reductase polypeptide to a test

procedure that allows the determination of whether a methionine synthase reductase gene is wild-type or mutant. For example, one could analyze the methionine synthase reductase genes of an animal by amplifying genomic DNA using the polymerase chain reaction, and then determining the DNA sequence of the amplified DNA.

By “probe” or “primer” is meant a single-stranded DNA or RNA molecule of defined sequence that can base pair to a second DNA or RNA molecule that contains a complementary sequence (the “target”). The stability of the resulting hybrid depends upon the extent of the base pairing that occurs. The extent of base-pairing is affected by parameters such as the degree of complementarity between the probe and target molecules, and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as temperature, salt concentration, and the concentration of organic molecules such as formamide, and is determined by methods known to one skilled in the art. Probes or primers specific for methionine synthase reductase nucleic acid preferably will have at least 35% sequence identity, more preferably at least 45-55% sequence identity, still more preferably at least 60-75% sequence identity, still more preferably at least 80-90% sequence identity, and most preferably 100% sequence identity. Probes may be detectably-labelled, either radioactively, or non-radioactively, by methods well-known to those skilled in the art. Probes are used for methods involving nucleic acid hybridization, such as: nucleic acid sequencing, nucleic acid amplification by the polymerase chain reaction, single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism (RFLP) analysis, Southern hybridization, Northern hybridization, in situ hybridization, electrophoretic

mobility shift assay (EMSA).

By “pharmaceutically acceptable carrier” means a carrier which is physiologically acceptable to the treated mammal while retaining the therapeutic properties of the compound with which it is administered. One exemplary  
5 pharmaceutically acceptable carrier is physiological saline. Other physiologically acceptable carriers and their formulations are known to one skilled in the art and described, for example, in Remington’s Pharmaceutical Sciences, (18<sup>th</sup> edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA.

By "substantially identical" is meant a polypeptide or nucleic acid  
10 exhibiting, over its entire length, at least 50%, preferably 85%, more preferably 90%, and most preferably 95% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length  
15 of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

By “identity” is meant that a polypeptide or nucleic acid sequence possesses the same amino acid or nucleotide residue at a given position, compared  
20 to a reference polypeptide or nucleic acid sequence to which the first sequence is aligned.

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin  
25 Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This

software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "substantially pure polypeptide" is meant a polypeptide that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is a methionine synthase reductase polypeptide that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure methionine synthase reductase polypeptide may be obtained, for example, by extraction from a natural source (e.g., a fibroblast) by expression of a recombinant nucleic acid encoding a methionine synthase reductase polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state.

Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides not only includes those derived from eukaryotic organisms but also those synthesized in *E. coli* or other prokaryotes.

By "substantially pure DNA" is meant DNA that is free of the genes

which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "transgene" is meant any piece of DNA that is inserted by artifice into a cell, and becomes part of the genome of the organism that develops from that cell. Preferably the coding region of the transgene is operably linked to one or more transcriptional regulatory elements, including a promoter (as defined below) that direct transgene expression. Such a transgene may comprise a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell that includes a DNA sequence that is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic mammals (e.g., rodents such as rats or mice) and the DNA (transgene) is inserted by artifice into the genome. Transgenic organisms also may include transgenic nematodes, such as transgenic *Caenorhabditis elegans*, which are generated by methods known to those skilled in the art.

By "knockout mutation" is meant an alteration in the nucleic acid sequence that reduces the biological activity of the polypeptide normally encoded therefrom by at least 80% relative to the unmutated gene. The mutation may,



without limitation, be an insertion, deletion, frameshift mutation, or a missense mutation. Preferably, the mutation is an insertion or deletion, or is a frameshift mutation that creates a stop codon.

By "transformation" is meant any method for introducing foreign molecules into a cell (e.g., a bacterial, yeast, fungal, algal, plant, insect, or animal cell). Lipofection, DEAE-dextran-mediated transfection, microinjection, protoplast fusion, calcium phosphate precipitation, retroviral delivery, electroporation, and biolistic transformation are just a few of the methods known to those skilled in the art which may be used.

By "transformed cell" is meant a cell (or a descendant of a cell) into which a DNA molecule encoding a methionine synthase reductase polypeptide has been introduced, by means of recombinant DNA techniques.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., a methionine synthase reductase polypeptide, a recombinant protein or a RNA molecule).

By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific, temporal-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' or intron sequence regions of the native gene.

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the

regulatory sequences.

By "conserved region" is meant any stretch of six or more contiguous amino acids exhibiting at least 30%, preferably at least 50%, and most preferably at least 70% amino acid sequence identity between two or more reductase family members, (e.g., between human methionine synthase reductase and human cytochrome p450 reductase). An example of a conserved region within these two reductases is the NADPH binding region (Fig. 4).

By "detectably-labeled" is meant any means for marking and identifying the presence of a molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, or a cDNA molecule. Methods for detectably-labeling a molecule are well known in the art and include, without limitation, radioactive labeling (e.g., with an isotope such as  $^{32}\text{P}$  or  $^{35}\text{S}$ ) and nonradioactive labeling (e.g., chemiluminescent or fluorescent labeling, e.g., fluorescein labeling).

By "antisense" as used herein in reference to nucleic acids, is meant a nucleic acid sequence that is complementary to the coding strand of a gene, preferably, a methionine synthase reductase gene. An antisense nucleic acid is capable of preferentially decreasing the activity of a mutant methionine synthase reductase polypeptide encoded by a mutant methionine synthase reductase gene.

By "specifically binds" is meant that an antibody recognizes and binds a human methionine synthase reductase polypeptide, but does not substantially recognize and bind other non-methionine synthase reductase molecules in a sample, e.g., a biological sample, that naturally includes protein. A preferred antibody binds to the methionine synthase reductase polypeptide sequence of SEQ ID NO: 2 (Fig. 3).

By "neutralizing antibodies" is meant antibodies that interfere with any

of the biological activities of a wild-type or mutant methionine synthase reductase polypeptide, for example, the ability of methionine synthase reductase to catalyze the transfer of a methyl group to methionine synthase-cobal(II)amin. The neutralizing antibody may reduce the ability of a methionine synthase reductase polypeptide to catalyze the transfer preferably by 10% or more, more preferably by 25% or more, still more preferably by 50% or more, yet preferably by 70% or more, and most preferably by 90% or more. Any standard assay for the biological activity of methionine synthase reductase may be used to assess potentially neutralizing antibodies that are specific for methionine synthase reductase.

By “expose” is meant to allow contact between an animal, cell, lysate or extract derived from a cell, or molecule derived from a cell, and a test compound.

By “treat” is meant to submit or subject an animal (e.g. a human), cell, lysate or extract derived from a cell, or molecule derived from a cell to a test compound.

By “test compound” is meant a chemical, be it naturally-occurring or artificially-derived, that is surveyed for its ability to modulate an alteration in reporter gene activity or protein levels, by employing one of the assay methods described herein. Test compounds may include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof.

By “assaying” is meant analyzing the effect of a treatment, be it chemical or physical, administered to whole animals, cells, or lysates, extracts, or molecules derived therefrom. The material being analyzed may be an animal, a cell, a lysate or extract derived from a cell, or a molecule derived from a cell. The analysis may be for the purpose of detecting altered protein biological activity,

altered protein stability, altered protein levels, altered gene expression, or altered RNA stability. The means for analyzing may include, for example, the detection of the product of an enzymatic reaction, (e.g., the formation of active methionine synthase or methionine as a result of methionine synthase reductase activity),

5 antibody labeling, immunoprecipitation, and methods known to those skilled in the art for detecting nucleic acids.

By “modulating” is meant changing, either by decrease or increase, in biological activity.

By “a decrease” is meant a lowering in the level of biological activity, as  
10 measured by inhibition of: a) the formation of enzymatically active methionine synthase-cob(III)alamin-CH<sub>3</sub> or methionine as a result of methionine synthase reductase activity; b) protein, as measured by ELISA; c) reporter gene activity, as measured by reporter gene assay, for example, lacZ/ $\beta$ -galactosidase, green fluorescent protein, luciferase, etc.; or d) mRNA, as measured by PCR relative to  
15 an internal control, for example, a “housekeeping” gene product such as  $\beta$ -actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In all cases, the decrease is preferably by at least 10% more preferably by at least 25%, still more preferably by at least 50%, and even more preferably by at least 70%.

By “an increase” is meant a rise in the level of biological activity, as  
20 measured by a stimulation of: a) the formation of methionine synthase-cob(III)alamin-CH<sub>3</sub> or methionine as a result of methionine synthase reductase activity; b) protein, as measured by ELISA; c) reporter gene activity, as measured by reporter gene assay, for example, lacZ/ $\beta$ -galactosidase, green fluorescent protein, luciferase, etc.; or d) mRNA, as measured by PCR relative to an internal  
25 control, for example, a “housekeeping” gene product such as  $\beta$ -actin or

glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Preferably, the increase is by at least 10%, more preferably by at least 25%, still more preferably by at least 75%, even more preferably by 2-fold, and most preferably by at least 3-fold.

By “alteration in the level of gene expression” is meant a change in gene activity such that the amount of a product of the gene, i.e., mRNA or polypeptide, is increased or decreased, or that the stability of the mRNA or the polypeptide is increased or decreased.

By “reporter gene” is meant any gene that encodes a product whose expression is detectable and/or quantifiable by immunological, chemical, biochemical or biological assays. A reporter gene product may, for example, have one of the following attributes, without restriction: fluorescence (e.g., green fluorescent protein), enzymatic activity (e.g., lacZ/ $\beta$ -galactosidase, luciferase, chloramphenicol acetyltransferase), toxicity (e.g., ricin A), or an ability to be specifically bound by a second molecule (e.g., biotin or a detectably-labelled antibody). It is understood that any engineered variants of reporter genes, which are readily available to one skilled in the art, are also included, without restriction, in the forgoing definition.

By “protein” or “polypeptide” or “polypeptide fragment” is meant any chain of more than two amino acids, regardless of post-translational modification (e.g., glycosylation or phosphorylation), constituting all or part of a naturally-occurring polypeptide or peptide, or constituting a non-naturally occurring polypeptide or peptide.

By “missense mutation” is meant the substitution of one purine or pyrimidine base (i.e. A, T, G, or C) by another within a nucleic acid sequence, such that the resulting new codon may encode an amino acid distinct from the

amino acid originally encoded by the reference (e.g. wild-type) codon.

By “deletion mutation” is meant the deletion of at least one nucleotide within a polynucleotide coding sequence. A deletion mutation alters the reading frame of a coding region unless the deletion consists of one or more contiguous 3-nucleotide stretches (i.e. “codons”). Deletion of a codon from a nucleotide coding region results in the deletion of an amino acid from the resulting polypeptide.

By “frameshift mutation” is meant the insertion or deletion of at least one nucleotide within a polynucleotide coding sequence. A frameshift mutation alters the codon reading frame at and/or downstream from the mutation site. Such a mutation results either in the substitution of the encoded wild-type amino acid sequence by a novel amino acid sequence, or a premature termination of the encoded polypeptide due to the creation of a stop codon, or both.

By “low serum cobalamin level” is meant a serum cobalamin concentration of less than 328 pmol/L in a child, fetus, or embryo that has a neural tube defect or is at risk for developing a neural tube defect, or a serum cobalamin concentration of less than 259 pmol/L in the mother or future parent of a child having a neural tube defect.

By “polymorphic methylenetetrahydrofolate reductase” or “mutant methylenetetrahydrofolate reductase” is meant methylenetetrahydrofolate reductase (MTHFR) polypeptide or nucleic acid having a sequence that confers an increased risk of a disease phenotype in at least some genetic and/or environmental backgrounds, for example, in combination with an MMTR polymorphism or mutation.

By “677C→T polymorphism in MTHFR” is meant a substitution of cytosine in place of thymine in nucleic acid encoding MTHFR at a nucleotide

position equivalent to MTHFR nucleotide position 677 as disclosed in Frosst et al. (*Nat. Genet.* 10:111-113, 1995) and in Genbank Accession No. U09806 (SEQ ID NO: 51).

By “future parent” is meant a male or female who has contributed or may potentially contribute genetic material (e.g., a sperm or an egg) to form a zygote. A future parent is also a female who gestates or may potentially gestate an embryo or fetus in her uterus, irrespective of whether she has contributed or may potentially contribute genetic material to the embryo or fetus; an example of such a future parent is a surrogate mother).

By “test subject” is meant a future parent as defined above, an embryo, or a fetus.

By “sample from a test subject” is meant a specimen, for example, and not limited to, blood, serum, cells, or amniotic fluid, that would allow one of skill in the art to determine whether the test subject has a mutant or polymorphic methionine synthase reductase.

#### Detailed Description of the Invention

Methionine synthase catalyzes the remethylation of homocysteine to methionine in a reaction in which methylcobalamin serves as an intermediate methyl carrier.

Over time, the cob(I)alamin cofactor of methionine synthase may become oxidized to cob(II)alamin, thus rendering the enzyme inactive. Regeneration of the functional enzyme occurs through the reductive methylation of the cob(II)alamin in a reaction in which S-adenosylmethionine is utilized as methyl donor (Fig. 1). The reductive activation system in the lower part of the

scheme shown in Fig. 1 is the mechanism by which S-adenosylmethionine (Ado-Met) together with an electron reactivates the enzyme to the functional, methionine synthase-CH<sub>3</sub>-Co(III) state, resulting in the formation of S-adenosylhomocysteine (Ado-Hcy) as a reaction by-product.

Patients of the *cblE* complementation group of disorders of folate/cobalamin metabolism, who are defective in the reductive activation of methionine synthase, have megaloblastic anemia, developmental delay, hyperhomocysteinemia, and hypomethioninemia. We have cloned a cDNA corresponding to the "methionine synthase reductase" reducing system required for maintenance of the methionine synthase in a functional state. Using primers comprising sequences of consensus binding sites for FAD, FMN and NADPH, we performed RT-PCR and inverse PCR to clone a methionine synthase reductase cDNA. The cDNA hybridizes to an mRNA of 3.6 kb (as detected by Northern blot). The deduced protein is a novel member of the FNR family of electron transferases, containing 698 amino acids with a predicted Mr of 77,700. It shares 38% identity with human cytochrome P450 reductase and 43% with the *C. elegans* putative methionine synthase reductase (see below). Methionine synthase reductase was localized to human chromosome 5p15.2-15.3 by fluorescence *in situ* hybridization (FISH).

A survey of the NCBI databases for homology to the human methionine synthase reductase using BLASTP or TBLASTN yielded the putative methionine synthase reductase of *C. elegans* (P value =  $9 \times 10^{-92}$ ). Proteins of the FNR family were also found using the BLAST programs. The strongest homology was found with cytochrome P450 reductase (P values  $> 3 \times 10^{-68}$ ), followed by nitric oxide synthase (three isoforms, P values  $> 4 \times 10^{-52}$ ), and sulfite reductase (P values  $>$



6x10<sup>-39</sup>). Lower, but still significant homology was found with *E. coli* NADPH-ferredoxin(flavodoxin) reductase (P values > 2x10<sup>-9</sup>) and flavodoxin (P values > 3x10<sup>-2</sup>). Our finding suggests a convergent evolution of the two-gene flavodoxin/NADPH-ferredoxin(flavodoxin) reductase system to a single gene encoding a fused version of the two proteins in human cells. Alignment of the proteins provides for a large linker region bridging the two components.

The identity of our cloned cDNA sequence as that encoding methionine synthase reductase was confirmed by the identification of mutations in the corresponding gene in *cblE* patients having a functional deficiency of methionine synthase. Our key finding confirming the identification of the cDNA was a 4 bp frameshift mutation in two affected siblings. The occurrence of a functionally null mutation in a candidate gene provides compelling evidence that the mutation is causative of disease in the affected patients. Furthermore, a 3 bp deletion detected in a third patient is also highly likely to cause an enzyme defect, and the direct sequencing of PCR products suggested that the patient's second allele contains a mutation that renders the mRNA very unstable or poorly transcribed. In all, seven of ten tested *cblE* cell lines showed evidence of mutation although the sequence changes have yet to be determined in the remaining four.

The two mutations we have identified associated with *cblE* disease are located in the vicinity of the NADPH binding domain by comparison with proteins of the FNR family. The 4 bp deletion yields a truncated protein that is expected to be deficient in NADPH binding and possibly in FAD binding, since the C-terminus of the enzyme may be involved in both. The 3 bp deletion results in the deletion of Leu576, which is located between two sequences that may be involved in NADPH binding. Leu576 is well conserved among reductases that are

similar to the methionine synthase reductase (Fig. 6C). This supports the idea that deletion of the Leu576 codon (1726delTTG) results in an enzymatic defect, although confirmation will require expression of the mutant protein. This residue is also conserved in the NADPH-ferredoxin (flavodoxin) reductase enzymes of several organisms, although the homology with this portion of the protein is low or absent in some cases. It is possible that the deletion affects the relationship between the two NADPH-binding sequences that are in its vicinity.

The cloning of human methionine synthase reductase cDNA enables the determination of the enzymatic mechanism involved in the reductive activation of methionine synthase. Furthermore, it is now possible to identify additional mutations in patients with severe deficiency of the enzyme activity, and to determine whether there exist common amino acid polymorphisms which lead to mildly elevated homocysteine levels. Such elevations may be a risk factor in cardiovascular disease, neural tube defects, and cancer.

Mutations in the human methionine synthase reductase gene that result in altered homocysteine and/or folate levels may be risk factors for the diseases listed above. The methods of the invention therefore provide diagnostic assays for such risk factors, as well as methods of treating or preventing cardiovascular disease, neural defects, cancer, megaloblastic anemia, and hypomethioninemia. In addition, the invention provides methods for screening assays for the isolation of potential therapeutic compounds that modulate methionine synthase reductase activity.

The assays described herein can be used to test for compounds that modulate methionine synthase activity and hence may have therapeutic value in the prevention of neural tube defects, prevention and/or treatment of cancer,

cardiovascular disease, homocysteinemia, and megaloblastic anemia.

## Test Compounds

In general, novel drugs for prevention of neural tube defects, or prevention and/or treatment of cancer, cardiovascular disease, and megaloblastic anemia are identified from large libraries of both natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their therapeutic activities for homocysteinemia, megaloblastic anemia, cardiovascular disease, cancer, and neural tube defects should be employed whenever possible.

When a crude extract is found to modulate methionine synthase reductase biological activity, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect.

Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude extract that modulates methionine synthase reductase biological activity. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogenous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value may be subsequently analyzed using mammalian models of homocysteinemia, megaloblastic anemia, cardiovascular disease, cancer, and neural tube defects.

Methionine synthase reductase assays for the detection of compounds that modulate methionine synthase reductase activity and expression

Potentially useful therapeutic compounds that modulate (e.g. increase or decrease) methionine synthase reductase activity or expression may be isolated by

various screens that are well-known to those skilled in the art. Such compounds may modulate methionine synthase reductase expression at the pre- or post-transcriptional level, or at the pre- or post-translational level.

A. Screens for compounds that modulate methionine synthase reductase enzymatic activity

Screens for potentially useful therapeutic compounds that modulate methionine synthase reductase activity may be readily performed. For example, the effect of a test compound on methionine synthase reductase activity may be determined by measuring formation of  $^{14}\text{CH}_3\text{-cob(III)alamin}$ , which results from the transfer of  $^{14}\text{CH}_3$  from S-adenosylmethionine to methionine synthase-cob(II)alamin. A test compound that increases the enzymatic activity of a methionine synthase reductase would result in increased levels of methionine synthase- $^{14}\text{CH}_3\text{-cob(III)alamin}$ , and a compound that decreases the enzymatic activity of a methionine synthase reductase would result in decreased levels of methionine synthase- $^{14}\text{CH}_3\text{-cob(III)alamin}$ .

The effect of a test compound on methionine synthase reductase activity also may be determined by measuring the resulting activity of methionine synthase. The amount of reaction product (i.e., methionine) formation reflects the relative activity of methionine synthase, which in turn reflects the relative activity of methionine synthase reductase, which in turn indicates the effect of the test compound on methionine synthase reductase activity. For example, a sample containing methionine synthase and homocysteine may contain a mutant, inactive methionine synthase reductase which does not reduce oxidized methionine synthase, and hence, no methionine is formed. However, a test compound that

increases the enzymatic activity of the mutant methionine synthase reductase will result in increased levels of methionine formation, relative to control samples not containing the test compound. Analogously, a compound that decreases methionine synthase reductase activity will result in the formation of decreased levels of methionine formation in reactions containing active methionine synthase reductase. That a test compound directly modulates methionine synthase reductase enzymatic activity, as opposed to methionine synthase enzymatic activity, can be confirmed by including control reactions that lack methionine synthase reductase. Such control reactions should not show altered levels of methionine production if the test compound directly modulates methionine synthase reductase activity.

Examples of methionine synthase activity assays, *in vitro* and in whole cells, are well-known to those skilled in the art (see, for example, Gulati et al., 1997, *J. Biol. Chem.* 272:19171-19175; see also Rosenblatt et al., 1984, *J. Clin. Invest.* 74:2149-2156).

#### B. ELISA for the detection of compounds that modulate methionine synthase reductase expression

Enzyme-linked immunosorbant assays (ELISAs) are easily incorporated into high-throughput screens designed to test large numbers of compounds for their ability to modulate levels of a given protein. When used in the methods of the invention, changes in a given protein level of a sample, relative to a control, reflect changes in the methionine synthase reductase expression status of the cells within the sample. Protocols for ELISA may be found, for example, in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997. Lysates from cells treated with potential modulators of methionine synthase

reductase expression are prepared (see, for example, Ausubel et al., *supra*), and are loaded onto the wells of microtiter plates coated with “capture” antibodies specific for methionine synthase reductase. Unbound antigen is washed out, and a methionine synthase reductase-specific antibody, coupled to an agent to allow for detection, is added. Agents allowing detection include alkaline phosphatase (which can be detected following addition of colorimetric substrates such as *p*-nitrophenolphosphate), horseradish peroxidase (which can be detected by chemiluminescent substrates such as ECL, commercially available from Amersham) or fluorescent compounds, such as FITC (which can be detected by fluorescence polarization or time-resolved fluorescence). The amount of antibody binding, and hence the level of a methionine synthase reductase polypeptide within a lysate sample, is easily quantitated on a microtiter plate reader.

As a baseline control for methionine synthase reductase expression, a sample that is not exposed to test compound is included. Housekeeping proteins are used as internal standards for absolute protein levels. A positive assay result, for example, identification of a compound that increases or decreases methionine synthase reductase expression, is indicated by an increase or decrease in methionine synthase reductase polypeptide within a sample, relative to the methionine synthase reductase level observed in cells which are not treated with a test compound.

#### C. Reporter gene assays for compounds that modulate methionine synthase reductase expression

Assays employing the detection of reporter gene products are extremely sensitive and readily amenable to automation, hence making them ideal for the



design of high-throughput screens. Assays for reporter genes may employ, for example, colorimetric, chemiluminescent, or fluorometric detection of reporter gene products. Many varieties of plasmid and viral vectors containing reporter gene cassettes are easily obtained. Such vectors contain cassettes encoding  
 5 reporter genes such as lacZ/ $\beta$ -galactosidase, green fluorescent protein, and luciferase, among others. Cloned DNA fragments encoding transcriptional control regions of interest (e.g. that of the mammalian methionine synthase reductase gene) are easily inserted, by DNA subcloning, into such reporter vectors, thereby placing a vector-encoded reporter gene under the transcriptional control of any  
 10 gene promoter of interest. The transcriptional activity of a promoter operatively linked to a reporter gene can then be directly observed and quantitated as a function of reporter gene activity in a reporter gene assay.

Cells are transiently- or stably-transfected with methionine synthase reductase control region/reporter gene constructs by methods that are well known  
 15 to those skilled in the art. Transgenic mice containing methionine synthase reductase control region/reporter gene constructs are used for late-stage screens *in vivo*. Cells containing methionine synthase reductase/reporter gene constructs are exposed to compounds to be tested for their potential ability to modulate methionine synthase reductase expression. At appropriate timepoints, cells are  
 20 lysed and subjected to the appropriate reporter assays, for example, a colorimetric or chemiluminescent enzymatic assay for lacZ/ $\beta$ -galactosidase activity, or fluorescent detection of GFP. Changes in reporter gene activity of samples treated with test compounds, relative to reporter gene activity of appropriate control samples, indicate the presence of a compound that modulates methionine synthase  
 25 reductase expression.

D. Quantitative PCR of methionine synthase reductase mRNA as an assay for compounds that modulate methionine synthase reductase expression

The polymerase chain reaction (PCR), when coupled to a preceding reverse transcription step (rtPCR), is a commonly used method for detecting vanishingly small quantities of a target mRNA. When performed within the linear range, with an appropriate internal control target (employing, for example, a housekeeping gene such as actin), such quantitative PCR provides an extremely precise and sensitive means of detecting slight modulations in mRNA levels. Moreover, this assay is easily performed in a 96-well format, and hence is easily incorporated into a high-throughput screening assay. Cells are treated with test compounds for the appropriate time course, lysed, the mRNA is reverse-transcribed, and the PCR is performed according to commonly used methods, (such as those described in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY, 1997), using oligonucleotide primers that specifically hybridize with methionine synthase reductase nucleic acid. Changes in product levels of samples exposed to test compounds, relative to control samples, indicate test compounds that modulate methionine synthase reductase expression.

Secondary screens of test compounds that appear to modulate methionine synthase reductase activity

After test compounds that appear to have methionine synthase reductase-modulating activity are identified, it may be necessary or desirable to subject these compounds to further testing. At late stages testing will be

performed *in vivo* to confirm that the compounds initially identified to affect methionine synthase reductase activity will have the predicted effect *in vivo*. Such tests may be performed using cells or animals that have wild-type, mutated, or deleted methionine synthase reductase genes, or wild-type or mutated methionine synthase reductase transgenes.

### Therapy

Compounds identified using any of the methods disclosed herein, may be administered to patients or experimental animals with a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer such compositions to patients or experimental animals. Although intravenous administration is preferred, any appropriate route of administration may be employed, for example, parenteral, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer,

lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for antagonists or agonists of the invention include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

The following examples are to illustrate, not limit the invention.

## EXAMPLE 1: GENERAL METHODS

### Materials

Radiolabeled compounds were from DuPont (Wilmington, DE). A human multiple tissue Northern blot and  $\beta$ -actin probe were from Clontech (Palo Alto, CA). The random-primed DNA labelling kit was from Boehringer Mannheim (Indianapolis, IN). The T/A cloning kit was from Invitrogen (Carlsbad, CA), the GeneClean III kit was obtained from Bio101 Inc. (Vista, CA), and the Wizard Mini-Preps were from Promega (Madison, WI). Taq polymerase, AMV reverse transcriptase, Trizol reagent, and were purchased from Gibco BRL (Gaithersburg, MD), and restriction enzymes were purchased from GibCo BRL and New England Biolabs (Beverly, MA). The Sequenase kits for manual sequencing of crude PCR products or plasmids were from United States Biochemicals (Cleveland, OH). The oligonucleotides (SEQ ID NOs: 3-20 and 49-50) were synthesized by ACGT Corporation (Toronto, Canada) or by the Sheldon

Biotechnology Centre, McGill University. The sequences of oligonucleotides are shown in Table 1 and in Fig. 2. A human cDNA library, made in Lambda-ZAP from RNA derived from the human colon carcinoma line Caco-2, was used as template in some PCR reactions to obtain 5' extensions of the cDNA.

## 5 Homology matches

Comparisons were made between putative FMN, FAD and NADPH binding sites and sequences in the NCBI databases (dbEST and nr) using the BLAST programs (Altschul et al., *Nat. Genet.* 6:119-129, 1994). The cytochrome P450 reductase and nitric oxide synthase full sequences were also used for  
10 homology searching.

## PCR cloning and DNA sequencing

Total cellular RNA was isolated by the method of Chirgwin et al. (*Biochemistry*, 18:5294-5299, 1979) and reverse-transcribed using oligo-dT15 as primer. PCR was conducted as described previously (Triggs et al., *Am. J. Hum.*  
15 *Genet.* 49:1041-1054, 1991). The PCR products were purified using GeneClean, subcloned in the pCR2.1 vector and transformed into *E. coli* according to the supplier's protocol (TA cloning kit). The resulting clones were sequenced manually to confirm the specificity of PCR products. Automated sequencing was done by Bio S&T Inc. (Montreal, Canada) or by the DNA Sequencing Core  
20 Facility of the Canadian Genetic Diseases Network.

## Northern blot

The multiple tissue Northern blot, prepared from poly(A)+ RNA (2

μg/lane) of the indicated human tissues, was probed with an EcoRI segment of a subclone in pCRII containing an insert spanning positions 335-2148 of the methionine synthase reductase cDNA. Hybridization with human β-actin cDNA served as a control for the quantity and integrity of the RNA in the blot.

## 5 Chromosomal localization

We performed PCR analysis of DNA from the NIGMS human/rodent somatic cells hybrid mapping panel (#2). The oligonucleotide primers, which were specific for the 3'-UTR region of the gene, amplified a 111 nucleotide product (accession #G19837 in dbSTS). A P1-derived artificial chromosome (PAC) clone (104K2) was identified from a total human genomic library (Ioannou, P.A. et al., *Nat. Genet.* 6:84-89, 1994) by hybridization screening with a methionine synthase reductase cDNA probe (clone 704947, accession #AA279726 in dbEST) and this genomic clone was then used for FISH mapping (Heng, H. H. et al., *Proc. Natl. Acad. Sci. USA* 89:9509-9513, 1992; Heng, H.H and Tsui, L.C., *Chromosoma* 102:325-332, 1993).

## Cell lines

Ten fibroblast cell lines from patients with homocystinuria (*cbIE* complementation group) were used to identify mutations and polymorphisms in the MTRR gene using reverse transcription-PCR of total cellular RNA. Three of the cell lines displayed mutations: WG788 from the original *cbIE* patient (Schuh et al., *N. Engl. J. Med.* 310:686-690, 1984); WG1146 from his younger brother, who had been diagnosed before birth, and whose mother was treated with hydroxocobalamin during pregnancy (Rosenblatt et al., *Lancet* 1:1127-1129,

1985); and WG1836 from a patient who had previously been described as having dihydrofolate reductase deficiency (case 1 in Tauro et al., *N. Engl. J Med.* 294:466, 1976) and subsequently as having a "new mutation" associated with low methylcobalamin levels and reduced cellular folate uptake (Brasch et al, *Aust. N. Z. J. Med.* 18 Supp.434, 1988). In our laboratory, we have shown that the fibroblast line from this last patient falls into the *cblE* complementation group.

The fibroblast cell line WG1401 was the first to show the polymorphism, an A to G substitution at bp 66. WG1401 is from patient B.S.S. 17, with megaloblastic anemia, hyperhomocysteinemia, and mild methylmalonic aciduria. The polymorphism was also found in a control cell line, MCH64.

Twenty-two other cell lines were used as normal controls for mutation analysis.

#### Mutation analysis by RT-PCR of fibroblast RNA

Total cellular RNA was isolated from fibroblast pellets (Chirgwin et al., *Biochemistry*, 18:5294-5299, 1979). It was reverse transcribed using 25  $\mu$ g total RNA in reactions containing 2.5 U of AMV reverse transcriptase and 500 ng of methionine synthase reductase-specific terminal oligonucleotide 2101C (SEQ ID NO: 20; Table 1) in a total reaction volume of 54  $\mu$ l. The resultant cDNA was used as template for PCR. PCR for nine overlapping cDNA segments was performed in reactions containing 3  $\mu$ l of template, 1  $\mu$ l each of dTTP, dGTP, dATP and dCTP (10 mM), and 3 U Taq polymerase in a 46  $\mu$ l volume. PCR products were verified by agarose gel electrophoresis before testing for heteroduplex formation. Heteroduplex analysis was carried out by mixing mutant and control PCR products 1:1, heating the mixture to 95°C for 3 min, cooling to

room temperature, and subjecting the samples to electrophoresis on an 8% polyacrylamide gel. Fragments displaying shifts were subcloned and sequenced, or sequenced directly.

#### MMTR polymorphism analysis in genomic DNA samples

For the screening of genomic DNA samples, restriction digestion analysis was performed with an artificially-created NdeI restriction site using the sense primer MSG108S 5'GCAAAGGCCATCGCAGAAGACAT (SEQ ID NO: 49) and antisense primer AD292 5'GTGAAGATCTGCAGAAAATCCATGTA (SEQ ID NO: 50), where the underlined C replaces the A to generate an NdeI restriction site in the normal sequence. To test for the mutation, 10  $\mu$ l of PCR product was digested by adding 6  $\mu$ l H<sub>2</sub>O, 2  $\mu$ l New England Biolab's (NEB) buffer 4 and 2  $\mu$ l NdeI. The PCR fragment of 66 bp remains uncut in the presence of the G (methionine) allele, but is digested into fragments of 44 bp and 22 bp in the presence of the A (isoleucine) allele.

#### Subjects

Patients with spina bifida (n=56) and mothers of children with spina bifida (n=58) were recruited from the Montreal Children's Hospital after approval of the protocol by the Institutional Review Board. The controls (n=97) were other outpatients who were having a venipuncture at the Pediatric Test Center, Montreal Children's Hospital, and who were with their mothers (n=89). Blood samples were obtained from mothers and children after appropriate consent. Exclusion criteria were syndromic neural tube disorder (NTD) cases, severe anemia, neoplastic disease, renal insufficiency and immunosuppressive therapy. Individuals who were



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taking vitamin supplements were also excluded. The methylenetetrahydrofolate reductase (MTHFR) genotypes and the levels of plasma homocysteine and serum cobalamin were previously determined in these subjects. The concentration of serum cobolamin was quantitated by routine methods, using an automated system and reagents from Ciba (Ciba Corning Diagnostics Corp., Medfield, MA).

To determine total homocysteine (tHey) levels in plasma, blood samples were drawn to Becton-Dickinson vacutainers containing sodium EDTA and kept on ice until plasma was separated. Plasma was separated by centrifugation for 5 min., removed, and cetrifuged again; the supernatant was collected and frozen at - 20°C until analysis. tHcy in plasma was determined by high pressure liquid chromatography as reported (Gilfix et al., *Clin. Chem.* 43:687-688, 1997). The tHcy adduct was detected by fluorescence after precolumn derivitization with the thiol-specific reagent 7-fluoro-benzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F)(Wako, USA).

To detect the MTHFR polymorphism, DNA was isolated from peripheral leukocytes by extraction with phenol-chloroform after cell lysis in a buffer containing Nonidet-P40 (Boehringer Mannheim, Mannheim, Germany) and stored at -20°C. The presence of the 677C→T polymorphism in MTHFR (SEQ ID NO: 51) was determined by PCR followed by restriction digestion with HinfI, as described (Frosst et al., *Nat. Genet.* 10:111-113, 1995).

### Statistics

Computer-assisted statistical analyses were carried out using SAS for Windows (Version 6.12). Standard summary statistics, analysis of variance, t-tests, calculation of odds ratios with associated confidence limits, and logistic regression

models were used where appropriate. Statistical significance was interpreted as p-values of  $p < 0.05$ .

## EXAMPLE II: CLONING OF THE HUMAN METHIONINE SYNTHASE REDUCTASE cDNA

5 More than 20 overlapping sequences homologous to the FAD and  
NADPH-binding domains of cytochrome P450 reductase were identified in an  
initial survey of the NCBI dbEST database using TblastN. We sequenced clones  
550341 (accession #AA085543), 704947 (accession #AA279726) and 31776  
(accession #R17835) to confirm the sequence of this part of the cDNA. Reprobing  
10 the NCBI databases with this sequence yielded a *C. elegans* sequence (accession  
#Z35595) containing binding sites for FMN, FAD and NADPH. We then used the  
*C. elegans* sequence to reprobe the dbEST database using TblastN and identified a  
human sequence (accession #AA192690, clone 628497) containing a putative  
FMN binding site similar to the one encoded by Z35595. We designed a sense  
15 primer based on the FMN binding region of AA192690 and antisense primers  
corresponding to the FAD/NADPH binding regions of the methionine synthase  
reductase candidate and amplified a sequence by RT-PCR using human fibroblasts  
as the source of RNA. Fig. 2 shows the overlapping clones and PCR fragments  
used to clone and sequence human methionine synthase reductase. The EST  
20 clones are shown as rectangles, the subsequences that were available from the  
dbEST database are shown as hatched boxes, and the PCR fragments are  
represented as lines. The oligonucleotide names are indicated below the arrows in  
Fig. 2 and are described in Table 1 below. The primer in parentheses designates a  
mispriming outcome that generated valid internal sequence. The letter "V" in

black boxes indicates primers annealing to the vector of the cDNA library used as a template for PCR. The presence of a triangle above a segment indicates that it contained a deletion of 154 bp (open triangle) or 26 bp (black triangle), likely caused by alternative splicing.

Table 1. Oligonucleotides used for cDNA cloning,  
mapping, and mutation detection.

	<b>Primers</b>	<b>Sequence</b>	<b>Location</b>
5	Z116 (SEQ ID NO: 3)	5'-CTCCTGCTCGAACATCTTCCTAAA	1318 - 1341
	Z117 (SEQ ID NO: 4)	5'-AATAGATAATCCCTATCCTTATGCC	1766 - 1742
	AD150 (SEQ ID NO: 5)	5'-CCCTGGCTCCTAAGATATCCATC	1544 - 1566
	AD151 (SEQ ID NO: 6)	5'-CGAACAACAAATTCTTTCCACTTACC	1573 - 1598
	AB191 (SEQ ID NO: 7)	5'-CAAGGTTGGTGAAGTCGCGTTG	-79 - -57
10	AA468 (SEQ ID NO: 8)	5'-ATGCCTTGAAGTGATGAGGAGGTTT	-13 - 12
	AB586 (SEQ ID NO: 9)	5'-TTCCTACAACATAGAGAGAACTC	1663 - 1686
	AB588 (SEQ ID NO: 10)	5'-TTGCACAAGGGCATCATGTACATC	1998 - 1975
	Z593 (SEQ ID NO: 11)	5'-AAACCTCCTCATCACTTCAAGGCAT	12 - -13
	Z594 (SEQ ID NO: 12)	5'-CTTGCACACGAATATGGTCTGGG	1370 - 1348
15	Z596 (SEQ ID NO: 13)	5'-TGGCATCACCTGCATCCTTGAGG	506 - 528
	Z597 (SEQ ID NO: 14)	5'-GATGTACCTGTAAATATTCTGGGGG	760 - 736
	1103A (SEQ ID NO: 15)	5'-AATCCACGGCTCAACCACAAGTTC	429 - 406
	1761 (SEQ ID NO: 16)	5'-CTCGAAATTAACCCTCACTAAAGGG	in Bluescript
	1803E (SEQ ID NO: 17)	5'-AACCCATACCGCAGGTGAGCAAA	278 - 256
20	1812B (SEQ ID NO: 18)	5'-TTTAGTACTTTTCAGTCAAAAAAGCTTAAT	2148 - 2120
	1902C (SEQ ID NO: 19)	5'-ATAAACGACTTCAAGAGCTTGGAGC	335 - 359
	2101C (SEQ ID NO: 20)	5'-AGGTTTGGCACTAGTAAAGCTGACT	2173 - 2149
	MSG108S (SEQ ID NO: 49)	5'-GCAAAGGCCATCGCAGAAGACAT	43-65
	AD292 (SEQ ID NO: 50)	5'-GTGAAGATCTGCAGAAAATCCATGTA	83-108

The sequence of the PCR products confirmed that our cDNA contained the putative FMN, FAD and NADPH binding sites. The 5' end of the sequence was obtained by PCR using a cDNA library as template, with antisense primers specific for the cDNA and a sense primer that anneals to the vector used to construct the library. The sequences generated by PCR were taken as error-free by comparison of the sequence of at least two, and usually three, independent PCR reactions.

The coding sequence of human methionine synthase reductase contains 2094 bp (SEQ ID NO: 1 and SEQ ID NO: 41) encoding a polypeptide of 698 amino acids (SEQ ID NO: 2 and SEQ ID NO: 42) in length. Fig. 3 shows the cDNA sequence (SEQ ID NO: 24) and deduced amino acid sequence of human methionine synthase reductase. The nucleotide residues are numbered on the left margin, the amino acids residues are numbered on the right margin, and the stop codon is indicated by three stars. The sequence has been deposited in the GenBank database, accession #AF025794.

The predicted MW of human methionine synthase reductase is 77,700. It shares 38% sequence identity (49% similarity) with human cytochrome P450 reductase (accession #A60557) and 43% identity (53% similarity) with the *C. elegans* putative methionine synthase reductase (accession #Z35595). Fig. 4 shows amino acid sequence comparisons among human methionine synthase reductase (HsMTRR), *C. elegans* putative methionine synthase reductase (CeMTRR) and human cytochrome P450 reductase (HsCPR). The amino acids residues are numbered on the right margin, and conserved residues are shown by stars under the sequence. Alignments of similar amino acids are dotted (A,G,S,T;; D,E,N,Q; V,L,I,M; K,R; and F,W,Y), and regions proposed to be involved in

binding of FMN, FAD or NADPH are shown above the sequences.

The first in-frame methionine residue is a candidate for the initiation codon. It is perfectly aligned with the first methionine of the *C. elegans* sequence, and the presence of a G at positions -3 and -6 places the sequence in good context for initiation of translation (Kozak, *J. Biol. Chem.* 266:19867-19870, 1991). A polyadenylation signal is present at positions 3135-3140. The poly(A) tail is added after position 3165, although we observed some clones with polyadenylation after residue 3157.

RT-PCR involving various pairs of primers allowed us to detect alternatively processed methionine synthase reductase mRNA, including one form with a deletion of 154 bp (nucleotides 129-282) and another lacking a 26 bp segment (-52 to -27), accounting for less than 20% and 40% of the mRNA, respectively.

### EXAMPLE III: EXPRESSION OF HUMAN METHIONINE REDUCTASE mRNA

A PCR product generated with primers 1902C (SEQ ID NO: 19) and 1812B (SEQ ID NO: 18) was subcloned and used to probe a Northern blot prepared from several human tissues.

Figs. 5A and 5B show a Northern blot analysis of methionine synthase reductase expression in human tissues, with the positions of the molecular size (kb) markers indicated at the left. The 1.8 kb probe hybridized to one predominant RNA species of 3.6 kb. Methionine synthase reductase appears to be expressed to some degree in all tissues tested and is particularly abundant in skeletal muscle. In addition to the 3.6 kb band, a 3.1 kb band and a faint 6 kb band were detected in

brain mRNA.

#### EXAMPLE IV: CHROMOSOMAL MAPPING OF THE HUMAN METHIONINE SYNTHASE REDUCTASE GENE

The methionine synthase reductase gene was localized to human  
5 chromosome 5, since the gene-specific primer pair amplified a PCR product of the  
expected size only from the GM10114 hybrid, which contains chromosome 5 as its  
only human material. Moreover, the DNA sequence we determined for the  
methionine synthase reductase gene matched markers AA002A03 and STSG444,  
which were also mapped by the NCBI consortium to chromosome 5 between  
10 markers D5S406-D5S478 and D5S406-D5S635, respectively (Hudson, T.J. et al.,  
*Science* 270:1945-1954, 1995). To determine the cytogenetic position of the gene  
on chromosome 5, we mapped a genomic PAC clone encompassing the gene using  
fluorescence *in situ* hybridization (FISH). Fig. 6 shows a summary of the FISH  
mapping of the methionine synthase reductase gene to human chromosome  
15 5p15.2-p15.3. Each dot represents a signal detected on human chromosome 5.  
The hybridization efficiency was 100%, and, among 100 mitotic figures examined,  
each result indicated that the gene was located on chromosome 5p15.2-p15.3. We  
propose MTRR as the gene name for methionine synthase reductase, since the  
methionine synthase gene has been named MTR.

#### EXAMPLE V: MUTATIONS OF THE METHIONINE SYNTHASE REDUCTASE GENE IN PATIENTS OF THE *cbIE* COMPLEMENTATION GROUP

To confirm the identity of the candidate cDNA as methionine synthase

reductase, patient cell lines from the *cbIE* complementation group were analyzed by RT-PCR-dependent heteroduplex analysis using nine RT-PCR reactions that yielded overlapping products, in order to cover the length of the candidate cDNA sequence. Patient samples were mixed with RT-PCR product from normal cells to  
5 ensure the availability of wild-type DNA, in order to enable the detection of heteroduplexes in samples in which the mutation might be homozygous. For samples yielding heteroduplexes, the analysis was repeated without prior mixing with wild-type DNA, in order to determine whether the relevant changes were heterozygous. Three cell lines showed typical heteroduplex patterns, one of them  
10 observed in overlapping RT-PCR fragments (Fig. 7A and 7B).

Figs. 7A and 7B show a mutation analysis of the methionine synthase reductase gene in *cbIE* patient cell lines. Fig. 7A shows the PCR products obtained with primers Z116 (SEQ ID NO: 3) and Z117 (SEQ ID NO: 4) from RT reactions with control sample (WT) and two *cbIE* cell lines, WG1146 and  
15 WG1836. The bands above the 449 bp amplification product result from heteroduplexes formed between DNA strands bearing different allelic sequences. The pattern observed for cell line WG1146 was also seen with cell line WG788 (the sibling of WG1146). Fig. 7B shows RT-PCR products amplified with primers AB586 (SEQ ID NO: 9) and AB588 (SEQ ID NO: 10) from a control sample and  
20 cell line WG1836. Heteroduplexes are observed above the 336 bp band for cell line WG1836.

The heteroduplex-containing samples were subcloned and sequenced and two mutations were identified. A heterozygous mutation present in fibroblast line WG788 is a 4 bp deletion, 1675del4, resulting in a frameshift that creates a  
25 nearby stop codon. The same mutation was observed in cell line WG1146 from



the brother of patient WG788. Direct sequencing of the PCR product using primer AD150 showed overlapping sequences starting at position 1675, consistent with the heterozygous presence of the 4 bp deletion.

5 The second heterozygous mutation, detected in cell line WG1836, is an in-frame deletion of 3 bp, 1726delTTG. It results in the loss of a highly conserved leucine at position 576 of the amino acid sequence.

Fig. 7C shows a sequence comparison among proteins of the FNR family in a part of the NADPH binding region in the vicinity of the leucine residue that is deleted in a *cbIE* patient (denoted by a triangle; MTRR is methionine synthase reductase; CPR is cytochrome P450 reductase; NOS is nitric oxide synthase; SR is sulfite reductase; and FNR is NADPH-ferredoxin(flavodoxin) reductase).

Primer AD151 (SEQ ID NO: 6) was used for direct sequencing of the WG1836 PCR product. In this case, the deletion of nucleotides 1726-1728 was clearly visible. There was only a very faint background contributed by the normal sequence, suggesting that a second, unidentified mutation in this cell line was associated with a very low level of steady-state mRNA.

#### EXAMPLE VI: HUMAN METHIONINE SYNTHASE REDUCTASE POLYMORPHISMS

20 We have identified two polymorphisms in methionine synthase reductase cDNAs. The first is a G/A polymorphism at nucleotide position 66, using the "A" of the initiator methionine as nucleotide position number 1 (see Fig. 3), which results in either an isoleucine or a methionine, respectively, at amino acid 22. The second polymorphism is a G/A polymorphism at nucleotide position

110, which results in either a tyrosine or a cysteine, respectively, at amino acid position 37. It is likely that additional methionine synthase reductase polymorphisms will be found, some of which will be associated with increased or decreased risks of disease.

## 5 EXAMPLE VII: A COMMON POLYMORPHISM IN METHIONINE SYNTHASE REDUCTASE AS A RISK FACTOR FOR SPINA BIFIDA

During screening for methionine synthase reductase (MTRR) mutations in patients with homocystinuria, we identified an A/G polymorphism at bp 66, which yields an isoleucine (22I) or a methionine (22M), respectively, at amino  
10 acid position 22 (Figure 8A). Since the presence of the methionine polymorphism at this position did not create or obliterate a naturally-occurring restriction site, a PCR-dependent diagnostic test was established that makes use of a modified sense primer to create a NdeI site in the isoleucine allele during the amplification reaction. The PCR product of 66 bp remains uncut in the presence of the  
15 methionine allele, but is digested into fragments of 44 and 22 bp in the presence of the isoleucine allele (Figure 8B). The cDNA sequence reported in Leclerc, et al., *Proc. Natl. Acad. Sci. USA*, **95**:3059-3064, 1998, contained the methionine codon.

The NdeI assay was used to assess allele frequencies in controls. The 22I/22M polymorphism was extremely common in our control adult population  
20 (mothers of control children, n=89). Forty-nine percent were heterozygous while 26% were homozygous for the methionine allele (Table 2). The allele frequency was 0.51 for the methionine variant. Similar frequencies were observed for control children. The controls in this study were white Caucasian individuals with French, British, and mixed European ancestry. Since the allele frequency is virtually

identical for the two variants, the designation of a "wild type" allele could not be ascertained based on frequency. However, this gene has significant homology with related FMN-binding proteins from other organisms, including the putative methionine synthase reductase from *C. elegans*, as well as sulfite reductases, nitric oxide synthases, cytochrome P450 reductases, and flavodoxins. The equivalent codon in these genes is isoleucine, leucine, or valine in 123 out of 130 entries in GenBank. None of the entries contained a methionine codon. Consequently, the ancestral human MTRR sequence is likely to contain the isoleucine codon (22I), with a subsequent mutation to methionine (22M).

In this study, 34% (19/56) of case (spina bifida) children and 36% (21/58) of case mothers were homozygous for the 22M polymorphism in MTRR, compared to 30% (29/97) of control children and 26% (23/89) of control mothers (Table 2). An increased risk for being a case (odds ratio (OR) 1.7, 95% confidence interval (CI) 0.67-4.6) or a case mother (O.R. 2.0, 95% CI 0.77-5.2) was observed when the homozygous mutant (M/M) genotype was present, but this increase was not statistically significant. Mother-child genotype pairs were also assessed for neural tube defect (NTD) risk to determine if the combination of mutant maternal and mutant child genotypes conferred a greater risk than either genotype alone; an increased risk was not observed. Homocysteine levels were not increased in individuals who were homozygous mutant for MTRR (Table 3).

*Synergistic interaction between MTRR genotype and cobalamin level influences the risk of NTD*

Case children had serum cobalamin levels (pmol/L) of  $487 \pm 250$  (n=55), whereas control children had serum cobalamin levels of  $535 \pm 339$  (n=95);

case mothers had serum cobalamin levels of  $298 \pm 186$  (n=59), whereas control mothers had serum cobalamin levels of  $350 \pm 135$  (n=88; p=0.05). We therefore asked whether the mutant MTRR genotype may have a greater impact on NTD risk when cobalamin levels are low. Table 4 shows the results of multiple logistic regression analysis, adjusted for age, to test this hypothesis. Having a cobalamin level in the lowest quartile of the control distribution was associated with a nonsignificant two-fold increase in risk for the case mothers (O.R. = 2.1; 95% CI = 0.86-5.2). There was no increase in risk for low cobalamin in the children.

However, the combination of homozygous mutant genotype and low cobalamin was associated with a significant 5-fold increase in risk for the mothers, compared to those without the M/M genotype and with cobalamin levels in the other 3 quartiles (O.R. = 4.8, 95% CI = 1.5 - 15.8). The risk for the children with this combination was also increased but statistical significance was not observed (O.R. = 2.5, 95% CI = 0.63 - 9.7). There was no increased risk for the mutant genotype combined with low folate. Because the MTRR genotype alone was associated with less risk, we speculate that genotype and cobalamin levels work in unison to produce increased risk for spina bifida in the case mothers and case children.

#### *Synergistic interaction between MTRR and MTHFR genotypes influences the risk of NTD*

The 677C→T polymorphism (SEQ ID NO: 51) in the methylenetetrahydrofolate reductase (MTHFR) gene converts an alanine to a valine residue in the enzyme (Frosst et al., *Nat. Genet.* 10:111-113, 1995). MTHFR catalyzes the synthesis of 5-methyltetrahydrofolate, the primary circulatory form of folate and the methyl donor in the remethylation of

homocysteine to methionine by methionine synthase. Several studies have demonstrated an increased frequency of the homozygous mutant (V/V) MTHFR genotype in children with NTDs and in their mothers (van der Put et al., *Lancet* 346:1070-1071, 1995; Whitehead et al., *Quart. J. Med.* 88:763-766, 1995; Ou et al., *Am. J. Med. Genet.* 63:610-614, 1996).

Table 5 shows the interaction between the MTRR genotype and the MTHFR genotype in NTD risk, as determined by multiple logistic regression analysis, adjusted for age. Using a genotype of either homozygous wild type or heterozygous for MTRR and homozygous wild type for MTHFR as the reference, a risk nearly five times as great is conferred to case children (O.R. = 4.9, 95% CI=1.1-21.8) and to case mothers (O.R.= 5.0 , 95% CI 0.8 - 31.3) when they are homozygous for both mutations. The risk for the combination of mutant genotypes is clearly higher than either mutant genotype alone, in both the cases and in their mothers.

Table 2. Frequency of MTRR genotypes in children with spina bifida (cases) and in case mothers.			
	I/I	I/M	M/M
Cases	9/56 (16%)	28/56 (50%)	19/56 (34%)
Controls	24/97 (25%)	44/97 (45%)	29/97 (30%)
Case mothers	10/58 (17%)	27/58 (47%)	21/58 (36%)
Control mothers	22/89 (25%)	44/89 (49%)	23/89 (26%)
O.R. for children, M/M vs. I/I = 1.7 (95% C.I. 0.67-4.6)			
O.R. for mothers, M/M vs. I/I = 2.0 (95% C.I. 0.77-5.2)			

Table 3. Homocysteine levels stratified by MTRR genotype. (tHcy (μmol/L))			
	I/I	I/M	M/M
	n	n	n
Children	7.7 ± 2.8 33	8.2 ± 3.3 72	8.2 ± 3.1 48
Mothers	9.7 ± 2.8 32	10.3 ± 4.7 71	9.4 ± 3.1 43

5	Table 4. Logistic regression analysis for NTD risk in children and mothers. Odds ratio > (95% C.I.)			
	MTRR Genotype	Cobalamin level	Children	Mothers
10	I/I or I/M	normal	1.0 (ref.)	1.0 (ref.)
	I/I or I/M	low	0.92 (0.37-2.3)	2.1 (0.86-5.2)
	M/M	normal	1.1 (0.46-2.5)	1.5 (0.56-4.1)
	M/M	low	2.5 (0.63-9.7)	4.8 (1.5-15.8)
Odds ratios are adjusted by age of children and mothers respectively. Low cobalamin refers to the lowest quartile of the control distribution; normal refers to the other 3 quartiles.				

15	Table 5. Logistic regression analysis for NTD risk in children and mothers. Odds ratio > (95% C.I.)			
	MTRR Genotype	MTHFR Genotype	Children	Mothers
	I/I or I/M	A/A	1.0 (ref.)	1.0 (ref.)

I/I or I/M	V/V	0.82 (0.18-3.7)	2.4 (0.69-8.3)
M/M	A/A	1.2 (0.34-4.5)	1.9 (0.61-5.7)
M/M	V/V	4.9 (1.1-21.8)	5.0 (0.80-31.3)
Odds ratios are adjusted by age of children and mothers respectively.			

EXAMPLE VIII: HUMAN METHIONINE SYNTHASE REDUCTASE  
MUTATIONS AND POLYMORPHISMS IN DISEASE

Alterations in metabolism of folates, homocysteine, methionine, vitamin B12, and S-adenosylmethionine are associated with diseases such as megaloblastic anemia and conditions such as hyperhomocysteinemia. In turn, hyperhomocysteinemia may be associated with a higher than normal risk for cardiovascular disease and neural tube defects. In addition, decreased folate levels may be predictive of a lower than normal risk for cancer.

DNA samples from patients having a disease or developmental defect, such as those mentioned above, are analyzed for mutations within the methionine synthase reductase coding region and/or transcriptional control regions, and serum folate, red blood cell folate, plasma homocysteine, and serum cobalamin levels are measured. Patient samples are compared to control samples.

The cloning of the methionine synthase reductase gene makes possible the determination of whether discrete mutations and polymorphisms in methionine synthase reductase nucleic acid confer an increased risk for, or in contrast, protection against, diseases and conditions such as cardiovascular disease, cancer, and neural tube defects, (those of skill in the art will understand that polymorphisms and mutations may either increase or decrease the relative risk of any given disease or developmental defect). This collection of data in turn makes possible the development of diagnostic assays that predict whether a subject has a higher than normal risk of developing a disease or of having offspring with developmental defects. An understanding of disease-enhancing or -protective mutations allows the development of therapeutics that appropriately modulate methionine synthase reductase activity.



What is claimed is:

1. Substantially pure nucleic acid encoding a mammalian methionine synthase reductase polypeptide.

2. The nucleic acid of claim 1, wherein said nucleic acid encodes a human polypeptide.

5 3. The nucleic acid of claim 1, wherein said nucleic acid has the sequence of SEQ ID NO: 1 or SEQ ID NO: 41, or degenerate variants thereof, and wherein said nucleic acid encodes the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 42.

10 4. A substantially pure nucleic acid that hybridizes at high stringency to a sequence found within the nucleic acid of SEQ ID NO: 1 or SEQ ID NO: 41.

15 5. The nucleic acid of claim 4, wherein said nucleic acid has a sequence complementary to at least 50% of at least 60 nucleotides of the nucleic acid encoding the methionine synthase reductase polypeptide, said sequence sufficient to allow nucleic acid hybridization under high stringency conditions.

6. The nucleic acid of claim 5, wherein said nucleic acid contains a mutation or a polymorphism, and wherein said nucleic acid encodes a mutant or polymorphic polypeptide or a fragment thereof.

20 7. The mutation of claim 6, wherein said mutation is a 4 base deletion starting from base 1675 of SEQ ID NO: 1.

8. The mutation of claim 6, wherein said mutation is a deletion of 3 bases starting from base 1726 of SEQ ID NO: 1.

9. A non-human animal wherein one or both genetic alleles encoding a methionine synthase reductase polypeptide are mutated.

5 10. The animal of claim 9, wherein one or both genetic alleles encodes a methionine synthase reductase polypeptide are disrupted, deleted, or otherwise rendered nonfunctional.

11. The animal of claim 9, wherein said polypeptide has a mutation associated with hyperhomocysteinemia.

10 12. The animal of claim 9, wherein said animal is a rodent or a nematode.

13. An antibody that specifically binds a methionine synthase reductase polypeptide.

15 14. A method of detecting the presence of a methionine synthase reductase polypeptide, said method comprising contacting a sample with the antibody that specifically binds a methionine synthase reductase polypeptide and assaying for binding of said antibody to said polypeptide.

15. A method for detecting sequence variants for methionine synthase

reductase in a mammal, said method comprising analyzing the nucleic acid of a test subject to determine whether said test subject contains a mutation or polymorphism in a methionine synthase reductase gene, wherein the presence of said mutation or said polymorphism is an indication that said animal has an increased or decreased likelihood of developing hyperhomocysteinemia, cardiovascular disease, neural tube defects, or cancer.

16. A method of treating or preventing cancer, cardiovascular disease, or neural tube defects in a subject, said method comprising inhibiting methionine synthase reductase biological activity in said subject.

17. A method of treating or preventing cardiovascular disease, said method comprising administering to the subject to a therapeutically effective dose of a metabolite or cofactor selected from the group: folate, cobalamin, S-adenosyl methionine, betaine, or methionine.

18. The method of claim 16 or 17, wherein said subject has been diagnosed as having a mutation or polymorphism in methionine synthase reductase.

19. A method of preventing neural tube defects, cancer, or cardiovascular disease, said method comprising:

a) detecting an increased risk of neural tube defects, cancer, or cardiovascular disease, wherein said detecting is by analyzing methionine synthase reductase nucleic acid from one or more test subjects selected from: a mammal; a

potential parent, either male or female; a pregnant mammal; or a developing embryo or fetus, wherein said analyzing is done by the method of claim D; and

b) exposing said mammal, said potential parent, said pregnant mammal, and/or said developing embryo or fetus to a therapeutically effective dose of a metabolite or cofactor selected from the group: cobalamin; S-adenosyl methionine; betaine; or methionine, wherein said exposing is via the administration of said dose to said mammal, said potential parent, said pregnant mammal, and/or said developing embryo or fetus.

20. A method for screening for a compound that modulates methionine synthase reductase biological activity, said method comprising the steps of:

a) contacting a sample containing mutated or polymorphic methionine synthase reductase with said compound, and

b) assaying for methionine synthase reductase enzymatic activity, wherein increased enzymatic activity indicates an inducer of methionine synthase reductase biological activity, and decreased enzymatic activity indicates an inhibitor of methionine synthase reductase biological activity.

21. A method for screening for a compound that modulates methionine synthase reductase biological activity, said method comprising the steps of:

a) contacting a sample with said compound, and

b) assaying for methionine synthase reductase expression, wherein increased expression indicates an inducer of methionine synthase reductase biological activity, and decreased expression indicates an inhibitor of methionine synthase reductase biological activity.

22. A method for detecting an increased risk of developing a neural tube defect in a mammalian embryo or fetus, said method comprising detecting the presence of a polymorphic methionine synthase reductase (MTRR) in a test subject, wherein said polymorphic MTRR contains a methionine instead of an isoleucine at amino acid position 22, wherein said test subject is a future parent of said embryo or said fetus, and wherein detection of a homozygous MTRR polymorphism in said future parent, said embryo, or said fetus, or detection of either a homozygous or heterozygous MTRR polymorphism in both future parents, indicates an increased risk of developing said neural tube defect in said embryo or said fetus.

23. The method of claim 22, wherein said polymorphic MTRR is detected by analyzing nucleic acid from said test subject.

24. The method of claim 23, wherein said nucleic acid is genomic DNA.

25. The method of claim 23, wherein said nucleic acid is cDNA.

26. The method of claim 23, wherein said nucleic acid contains a G instead of an A at the third position of the twenty-second codon (nucleotide position 66, relative to the first nucleotide of the start codon) of MTRR.

27. The method of claim 23, said method further comprising:

a) PCR-amplifying a segment of MTRR nucleic acid using

primers MSG108S (SEQ ID NO: 49) and AD292 (SEQ ID NO: 50), and

b) digesting the product of the PCR amplification reaction with the restriction enzyme *Nde* I, wherein a PCR product that is digested by *Nde* I indicates an increased risk of developing a neural tube defect in a mammalian embryo or fetus.

28. The method of claim 22, wherein said polymorphic MTRR is detected by analyzing MTRR polypeptide from said test subject.

29. The method of claim 22, wherein said test subject is a future female parent of said embryo or said fetus.

30. The method of claim 22, wherein said test subject is said embryo or said fetus.

31. The method of claim 22, said method further comprising detecting the presence of a polymorphic methylenetetrahydrofolate reductase (MTHFR) in a test subject, said polymorphic MTHFR having a T instead of a C at a nucleotide position equivalent to position 677 of SEQ ID NO: 51, wherein detection of said polymorphic MTHFR indicates an increased risk of developing said neural tube defect in said embryo or said fetus.

32. The method of claim 31, wherein said polymorphic MTHFR is detected by analyzing nucleic acid from said test subject.

33. The method of claim 31, wherein said polymorphic MTHFR is detected by analyzing polypeptide from said test subject.

34. The method of claim 22, said method further comprising measuring the level of cobalamin in said test subject, wherein a low cobalamin level indicates  
5 an increased risk of developing said neural tube defect in said embryo or said fetus.



HUMAN METHIONINE SYNTHASE REDUCTASE: CLONING, AND  
METHODS FOR EVALUATING RISK OF NEURAL TUBE DEFECTS,  
CARDIOVASCULAR DISEASE, AND CANCER

Abstract of Invention

The invention features a novel gene encoding methionine synthase reductase. The invention also features a method for detecting an increased likelihood of hyperhomocysteinemia and, in turn, an increased or decreased likelihood of neural tube defects, cardiovascular disease, or cancer. The invention  
5 also features therapeutic methods for treating and/or reducing the risk of cardiovascular disease, cancer, or neural tube defects. Also provided are the sequences of the human methionine synthase reductase gene and protein and compounds and kits for performing the methods of the invention.

50004.003003 application.wpd

The diagram illustrates the methionine cycle, a metabolic pathway involving the conversion of methionine to homocysteine and back to methionine. The cycle is shown as a closed loop with two main enzymatic steps:

- Methionine Synthase:** This enzyme catalyzes the conversion of **Methionine** (labeled as  $(\text{CH}_3\text{-Homocysteine})$ ) to **Homocysteine**. This step is coupled with the oxidation of **MetSyn-Co(I)** to **MetSyn-Co(III)**. The cofactor **MetSyn-Co(III)** is shown with a methyl group ( $\text{CH}_3$ ).
- Methionine Synthase Reductase:** This enzyme catalyzes the conversion of **Homocysteine** back to **Methionine**. This step is coupled with the reduction of **MetSyn-Co(III)** back to **MetSyn-Co(I)**. The cofactor **MetSyn-Co(III)** is shown with a methyl group ( $\text{CH}_3$ ).

The diagram also shows the conversion of **Ado-Met** to **Ado-Hcy** via **Reductive Activation**, which is coupled with the oxidation of **MetSyn-Co(I)** to **MetSyn-Co(III)**. The cofactor **MetSyn-Co(III)** is shown with a methyl group ( $\text{CH}_3$ ).

1. The first part of the document is a title page. It contains the title of the document, the author's name, and the date of the document. The title is "The History of the United States of America". The author is "John Adams". The date is "1776".

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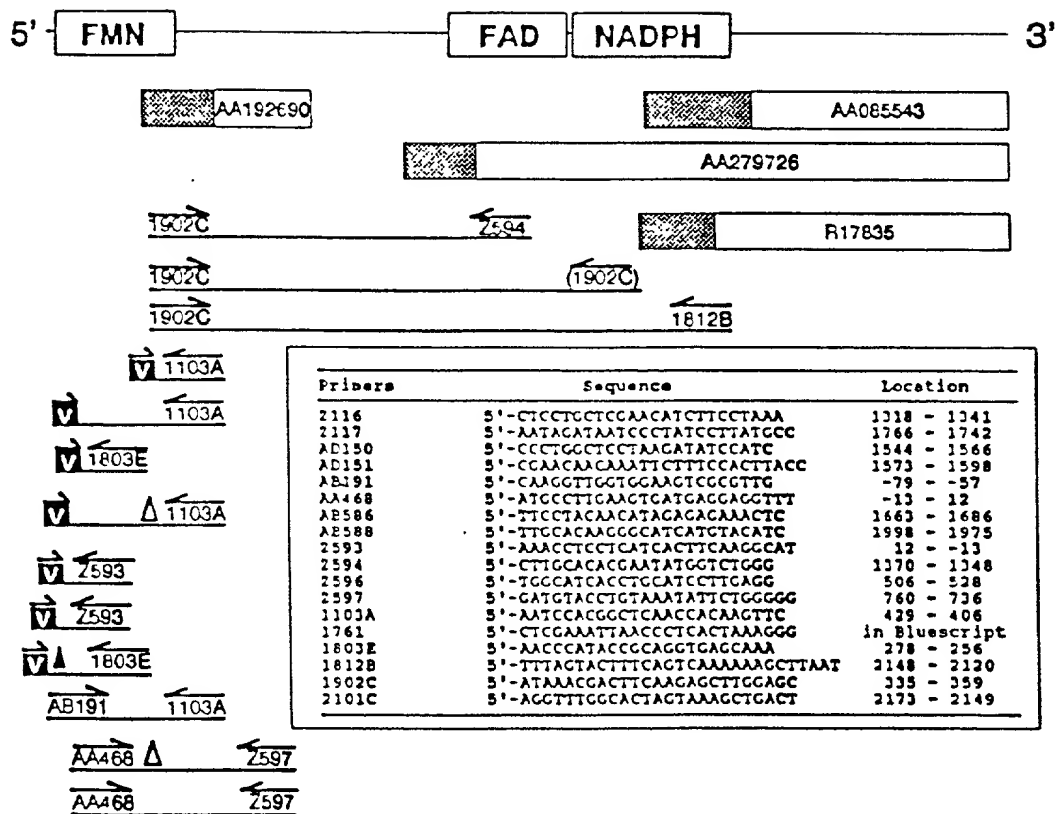


FIG. 2

CAAGTTCGTCGAAGTCGGTGTGCAGGTTCTGTCGCCCGCTGGCGCGCGTGGTTTCACTGTACATGCCCTGAAGTG

1 ATGAGCAGGTTTCTGTTACTATATGCTACACAGCGCCACGCGAAGCCATCGCAGAGAAATGTGTGAGCAAGTCTGGTACATCGATTCTTCCAGATCTTCACTGTATTAGTGAA  
M R R F F L L L Y A T C C G G A A C C G C C A C G G A A G C C A T C C G C A G A A A T G T G T G A G C A A G T C T G G T A C A T G G A T T C T T C C A G A T C T T C A C T G T A T T A G T G A A 40

121 TCCGATAGTATGACCTAAACCTCCAAACAGCTCTCTCTGTGTGTGGTTTCTACACAGCGGACCGGACCCAGCAGCAGCCGCAAGTTGTGTAAGAAATACAGAACCAACA  
S D K Y D L K E T T E T A P L V V V V S T T G T G D C P P D T A R K K F E I Q N O T 80

241 CTGCGGCTGATTCTTCTGTCACCTCGGGTATGGGTACTGGGTCTCGGTGATTGAGAATACACCTACTTTTGAATGGGGGAGAGATAATGATAAACGACTTCAAGCACTTCGAGCC  
L P V D T F F A H L R Y G L L G L G D S E Y T Y F C N G G K I I D K R L Q E L G A 120

361 CGGCATTTCATGACACTGGACATGCAGATCACTGTGTAGGTTTGAACATTGTGGTTCAGCGCTGGAATGCTGGCACTTCGGCCAGCTTCAGAAAGCAATTTAGGTCAAGCAGGACAA  
R H F Y D T G H A D D C V G L E L V V E P W I A G L W P A L R K H F R S S R G G 160

481 GAGGACATAAGTGGCGCACTCCGGTGGCATCAGCTGCTTGGAGGACAGCACTTGTGAAGTCAGAGTGGCTACACATTTGAATCTCAAGTCGAGCTTCTGAGATTTCGATGATTGAGGA  
E E I S G A L P V A S P A S L R T D L V K S E L L H I E S O V E L L R F D D S G 200

601 AGAAAGGATTCTGAGGTTTGAAGCAAAATCGACTGAACAGCAACCAATCCAATTTGTAATTGAAGCACTTGAAGTCTTCACTTACCGGTTTCGGTACCCCACTTCAACAGGCTCTCTG  
R K D S E V L K O N A V N S N Q S N V I E D F E S S L T R S V P L S O A S L 240

721 AATATTCTCGGTTTACCCCGCAATATTACAGGTACATTCGAGGAGTCTCTTGGCCAGGAGGAAACCAAGTATCTGTGACTTCAGCAGATCCAGTTTTCAAGTGCCTTTCAAG  
N I P G L P P E Y L O V H L Q E S L G O E S O V S V T S A D P V F O V P I S K 280

841 GCAGTTCAACTTACTACGAATGATGCCATAAAAAACACTCTGCTGGTAGAATTTGCAATTTCAATACAGACTTTTCTATCAGCTCGGATGCCCTCAGCGTGATCTGCCCTAACAGT  
A V L T T N D A I K T T L L V E L D I S N T D F S Y O P G D A F S V I C P N S 320

961 GATTCTGAGGTCAAGAGCTTACTTCAAAGCTTCAGGTTGAAGATAAAAGAGCACTGGCTCTTTGAAATTAAGCGACACACAAAGAGAGGAGTACCTTACCCCGCATATA  
D S E V O S L L O R L Q L E D K R E H C V L L K I K A D T K K K G A T L P O H I 360

1081 CTGCGGGATGTTCTCTCCAGTTCATTTTACCTGGTGCTTGAATTCGAGCAATTCCTAAAAGGCCATTTTTCGAGCGCCCTTGGCACTATACCACTGACAGTCAAGTCTGAAAGCGCGAGG  
P A G C S L O F I F T W C L E I R A I P K K A F L R A L V D Y T S D S A E K R R 400

1201 CTACAGGAGCTGTGCAGTAACAAAGCGGCGAGCCGATTATAGCCGCTTGTACAGCAAGTCCCTTGGCTGCTTGGGATCTTCCCTCGGTTTCCCTTCTTGGCAGCCAGCACTCAGTCTC  
L O E L C S K G A D Y S R F V R D A C A C L D L L L A F P S C O P P L S L 440

1321 CTGCTCGCAACTCTCTCTAACTTCAACCCAGACCATATTCTGTGCAAGTCAAGTTTATTTACCCAGGAAAGCTCCATTTTGTCTTCAACATTTGGGAATTTCTGTCTACTGCCACA  
L L E H L P K L O P R P Y S C A S S L F H P G K L H F V F N I V E F L S T A T 480

1441 ACAGAGGTTCTCGGCAAGGGAGTATGTACAGGTTGGTGGCTTGTTCGTTGCTTCAGTCTTCAGCCAAACATACATGCATCCCATGAAGCAAGCGGGAAAGCCCTGGCTCTAAGATA  
T E V L R K G V C T G W L A L L V A S V L O P N I H A S H E D S G K A L A P K I 520

1561 TCCATCTCTCTCGAACAACAAATTTCTTCCATTTACCACTGACCCCTCAATCCCATCAATAATGGTGGTTCAGGAACCGCATCCCGGTTTATTCGGTCTCTCAACATACAGAG  
S I S P R T T N S F A H L P D P S I P I I H V P P G T G I A P F I G F L O H R E 560

1681 AAATCTCAAGAACACACCCAGATGCAAAATTTGGAGCAATGTGGTGTGTTTTCGCTCGAGGCAATAGGATAGGATATCTATTCAAGAAAGAGCTCAGACATTTCTTAAAGTACGGG  
K L O E O H P D G N F G A M W L F F G C R H K D R O Y L F R K E L R H F L K H G 600

1801 ATCTTAACCTCATCAAGGTTTCTCTCAAGACATGCTCTGTTGGGAGGAGGAGGCCACGCAAGTATGTACAGAACACATCTCAGCTTCACTGGCCAGCAGGTGGCGAGATCTCTC  
I L T H L K V S F S R D A P V G E E A P A K Y V O D N I O L H G Q O V A R I L 640

1921 CTCGAGGAGACGGCCATATTATGTGTGTGCAGATCGAAACAATATGGCAAGGATGTACATGATGCCCTTGTGCAATAATAAGCAAGAGGTTGGAGTTGAAAACTAGAGCAATG  
L O E N G H I V C G D A K N H A K D V H D A L V Q I I S K E V G V E K L E A M 680

2041 AAAACCTTGGCCACTTTAAAGAGAGAAAACGCTACCTTCAGGATATTGGCTCAATAAACAGCAAAATTAAGAAAGAGGATTAAAGCTTTTGTAGTCAAGAGTCAAAAGCTCAGCTTTAC  
K T L A T L K E K R Y L D I W S \*\*\* 698

2161 TAGTGCCAAACCTTTAAATTTTCAAAAGAAATTTTCTTTCAACATTTCTGAGGACATGGAGTGGAGATTGGATCATTTAAACAATAACAACAACTTCTGATTGTATTTACGTATC  
2281 TTTCTATCAGCCCTTCTCTGCTGTGACITCTCCCAAAATTCGCTTGTGCTTGAGCTCTCTGAGCTAAAGGAGCGCTTCAGTCCCTATCAGCGCTCTCTTACTTCCAGAGCACT  
2401 TCACAGACACTCTGCTCTTCATCGAAAGGCTCTGCAATAGGGGACAGTCACTGAGTACATCTTTGTCAGCTACAGCTGCCACATTTAAAGAAATGTGAAATGATTATTTTGA  
2521 TATGATGTATACCCATAAAGAAATGCTCATTTAATGTACTTAAATACACATGTAGACAGATCTGTGTATGTTTATGTAACTATCAAAATGGTTATTTGTCTAAGGCTATTTTCTG  
2641 AAAAAAATATTTTAGGATAATTCCTCAAGAGGAGTTTATTTTATGATGCTGGAAATATGAAATGTAATTTAAAAATTTCACTCTGGGCATATGATTATCTATCAGCATCTGTTTCTG  
2761 TTTTAAAGTCAAAATTTTCAAGATTTTGGGACATTTTGCATTTCAATTTACAGGTACCACTAGCTACATTTTAAATAGAAAGATCAACCTTTTATTTTCTACCTCTTTTATTTCTGCTCT  
2881 GGCACTATTTTGTGTTTTCACATTTTGTCTCCATGATACCACTCAAGCAGTGTGCTGGACCTAAAACTAGCTTGTAGTATGATCTCTGGATTTTATGATTCCCAGTGTCTAAT  
3001 TCCCTGTTATAATTTGCACAAACAAACAAATTTATGATAATCTTCTCCACTCTTCTAATATATATGTATTTTATTTGATAGCTTGGGATTTAAACATCTCTGTTGAAGGCTTT  
3121 TGATCTCTTTGAGAAATAAAGATCTGAAGAAATGGCATTAATCTTAAAAAATAAAAAA



HsMTRR	-----	
CeMTRR	-----	
HsCPR	MGDSHVDTSSTVGEAAVEVSLFSMTDHLFSLIVGLLTYVFLFRKXXEE	50
FNN		
HsMTRR	-----HRRFLLLTATGCCAXATAEENCE	24
CeMTRR	-----HTDFLIATGSGTGAETIAKSLKE	24
HsCPR	VFEFTXICTLTSSVRESSFVEKMKKTGRNIIVFYCSGTGAEEFANRLSK	100
FNN		
HsMTRR	CAVVHGTADLHCISESDK-YDLKT-----ETAPLVVVVSTIGTGPP	66
CeMTRR	XAEITGLTFRHALCENEKKFNLE-----EKLC-AIVVSTIGTGPP	66
HsCPR	DAHRYG---HRCMSAGPEEYDLADLSSLEIDNALVVICMATYCEGQPT	146
FNN		
HsMTRR	DTAAKFXKEICMCTLPVDFFAHLRTGLGLGDSYTTFCNGCKIIDKRLQ	116
CeMTRR	ENCAFFVFRINRNELENEYLNLDYLLGLGDSNYSTGTIPRKIDKGLT	116
HsCPR	DNACQFYDULCETQVD--LSGVKFAVGLGNKTYENFAMGRYVCKRLE	193
FNN		
HsMTRR	ELGARHFDYDCHADDCVGLVVEPVIAGLWFAIRKHFSSRGQEEISGA	166
CeMTRR	ALGANRLDRAEADOCVGLLEVEPVIKFFATLASRFDISACKM----	162
HsCPR	QLGACRIFELCLGDCGCLLEDFITURECFWPAVCEHF-----GV	233
FNN		
HsMTRR	LPVASFASRLDTLVKSELLHIESOVELL--RFDSCGRKDSVLKCNVNS	214
CeMTRR	-AITESSNLKNCVXTE---EKKALLCKRIEEDSDEGRGVIGID-	206
HsCPR	EATGEESIKCYEL-----VVHTDGAARVYNGEMGLKSTEN	271
FNN		
HsMTRR	NCSNVVIEDF---ESSLTRSVPLS-CASLWIPGLPPETLOVHLOESLGO	260
CeMTRR	--MLIFEHTDYFESLLKCSQTLNDENLRVPIAPCFIVSSVSNKLP	253
HsCPR	CKP-----FFD-----AKNPFLLAATINRKLN	293
FNN		
HsMTRR	EESCUS-----VTSADPVCQVPIKAVOLT--NDAIKTLLVLELIS	301
CeMTRR	EDTKLEWNLCKMFQVTKFVFLVVSAEFVDPFSKKIKTKHITVDFG	303
HsCPR	GLTE-----RHLHLELD	306
FNN		
HsMTRR	N--TDFSYOFCCAFSVICPMSDSEVSSLOR-LOEDKREHCVLLKIKAD	348
CeMTRR	DHAELCYEFGDAITYFCVNPALVNFILKRCGLDIDAGCCEL-SINPK	352
HsCPR	ISCKIRYESCGHVAVYFANDSALVNLGK---ILGADLD--VVMSLNHL	351
FNN		
HsMTRR	TKXKCATLPCHPAGCSLQIFITWCLEIRAIPKXAFRLALVDYTSDAEK	398
CeMTRR	TEKINACIFGCHVKITLLRHHFITLDIRAFGRPLIRVLAESTSDPNEK	402
HsCPR	DEESKXKF--FPCPTSYRTALTYLDTNPPRTNVLVELACYASEFSEQ	399
FNN		
HsMTRR	RPLOEL--CSKCGAADYSRFRDACAQLDOLLAFSPCOPPLSLLLEHL	446
CeMTRR	RPILLE--CSACMKDFTDVTRTGLSLACMLFAFPNVKPPVORLIELLP	450
HsCPR	ELLKRMASSSGCKELYLSUVVEARRHILALDCCPSLRPPIDHCELLP	449
FAD		
HsMTRR	KICPRTYSCASSLHFPGKLFVFNIVEFLSTATTEVLKQVCTGWLALL	496
CeMTRR	RLIPRFTYSSSS---YENRKRLITYSEMFATGGRHRSKGLATDVLNLSL	497
HsCPR	RLCARTYSIASSSKVHPNSVHICAVVVEYTKAGR--INXGVATNUL---	496
FAD		
HsMTRR	VASVLPNINASHEDSCKALAPKISISPRITNSFHLN-----DOPSTP	539
CeMTRR	R-----IGDKVVLGKEFARFLPPLGTMXNSAGKLP	529
HsCPR	RAKE-----PVGCGGRALVPMFVRKSGFLPFK-----ATT	527
NADPH		
HsMTRR	IIMVGPCTGIAPFICFLCHREKLEOHDPGNFGAMU-LFFGCRHKDRYL	588
CeMTRR	LLMVGPCTGVSVFLSFLHFLRLKQDSFSDVVDVPRVLFEGCROSSVDAL	579
HsCPR	VIMVGPCTGVAFFIGIERAWLRGGKE---VGETLLTYGCRSSDEDYL	576
NADPH		
HsMTRR	FRKELRHFLXHGILTHLXVSFSDAPYGEAEAFKTYVDNITOLHGOQVAR	638
CeMTRR	YHSELEMFYSEGILTDLICESE-----XGERVQDGLRKYLDKVL	621
HsCPR	YFEELACFHRDGAITOLNVAFSRE-----QSHKVTVQHLKQDREHLWK	618
NADPH		
HsMTRR	ILLOE-NGHITVCGDAKMHAKDVHDLVQIISKEVGVKLEAMKTLATLK	687
CeMTRR	FLTASTESKIFICGDAXKSKDVVQCFSDIVASDGGIPOLEAKKKLMOLK	671
HsCPR	LI--EGGAHITVCGDARNMARDVGNFTFYDAELGAMEHAGAVDTIKKLM	666
NADPH/FAD		
HsMTRR	EEKRYLQDIWS	698
CeMTRR	KSDQYIEDVWG	682
HsCPR	TKGRYSLDWS	677

FIG. 4 page 1 of 2

HsMTRR	MGDSHVDTSSTVSEAVAEVSLFSMTDMILFSLIVGLLTYWFLRKKKEE	50
CeMTRR		
HsCPR		
	FMN	
HsMTRR	-----MRRFLLLYATOGGOAKAJAEEMCE	24
CeMTRR	-----MTDFLIAGSQTGOAETIAKSLKE	24
HsCPR	VPEFTKIOTLTSSVRESSFVEKMKKTGRNIIVFYGSQTGTAEFANRLSK	100
	.. . . . *	
	FMN	
HsMTRR	QAVVHGFSADLHCISESDK-YDLKT-----ETAPLVVVSTTGTGDP	66
CeMTRR	KAELIGLTPRLHALDENKKFNLNE-----EKLC-AIVVSTGDDGAP	66
HsCPR	DAHRYG---MRGMSADPEEYDLADLSSLPEIDNALVVFCMATYGECDPT	146
	* * . . . . *	
	FMN	
HsMTRR	DTARKFVKEIQNOTLPVDFFAHLRYGLGLGDSSEYTYFCNGGKIIDKRLQ	116
CeMTRR	DNCARFVRRIRNRSLENEYLNLDYVLLGLGDSNYSSYQTIIPRKIDKOLT	116
HsCPR	DNAQDFYDWLQETDVO---LSGVKFAVFLGNKTYEHFNAMGYVDKRL	193
	* * .. . . . *	
	FMN	
HsMTRR	ELGARHFYDTGHADDCVGLLVVEPWIALGLPALRKHFRSSRGQEEISGA	166
CeMTRR	ALGANRLFDRAEADDCVGLLEVEPWIEKFATLASRFDISADKMN----	162
HsCPR	OLGAORIFELGLGDDGNLEEDFITWREQFWPACVCEHF-----GV	233
	*** .. . . *	
	FMN	
HsMTRR	LPVASPASLRDVLKSELLHIESQVELL--RFDDSGRKDSEVLKONAVNS	214
CeMTRR	-AITESSNLKNOVKTE---EEKKALLQKRIEDESDEGRGRVIGID-	206
HsCPR	EATGEESSIRQYEL-----VVHTDIDAQVYMGEMGRKLSYEN	271
	. . . . .	
	FMN	
HsMTRR	NCSNVVIEDF---ESSLTRSVPLS-QASLNIPGLPPEYLOVHLOESLQ	260
CeMTRR	---MLIPENHYDPEISLLKGSQTLNDENLRVPJAPQPFIVSSVNRKLP	253
HsCPR	QKP-----PFD-----AKNPFLAAVTINRKLN	293
	. . . . .	
	FMN	
HsMTRR	EESQVS-----VTSADPVFOVPISKAVOLTT--NDAIKTLLVELDIS	301
CeMTRR	EDTKLEWONLCKMPGVVTKPFEVLVSAEFVTDPFSSKIKTKRMITVDFG	303
HsCPR	QGT-----RHLMHLELD	306
	. . . . .	
	FMN	
HsMTRR	N--TDFSYOPGDAFSVICPNSDSEVQSLLOR-LOLEDKREHCVLLKIKAD	348
CeMTRR	DHAAELQYEPDAIYFCVPNPALVNFILKRCGLVDIADQOCEL-SINPK	352
HsCPR	ISDSKIRYESGDHVAVYPANDSALVNOLGK---ILGADLD--VVMSLNNL	351
	. * . . . . *	
	FMN	
HsMTRR	TKKKGATLPQHIPAGCSLQFIFTWCLEIRAIPKKAFLRALVDYTSDAEK	398
CeMTRR	TEKINAQIPGHVHKITTLRHMTTCIDIRRAPGRPLIRVLAESTSDPNEK	402
HsCPR	DEESNKKHP--FPCPTSYRTALTYLDITNPRTNVLYELAQYASEPSEQ	399
	* . . . . *	
	FMN	
HsMTRR	RRLOEL--CSKOGAADYSRFVRDACACLDLLAFSPCOPPLSLLEHLP	446
CeMTRR	RRLEL--CSAOGMKDFTDFVRTPGLSLADMLFAFPNVKPPVDRLIELLP	450
HsCPR	ELLRKMASSSGEGKELYLSWVVEARRHILAILQDCPSLRPPIDHLCCELL	449
	* . . . . *	
	FAD	
HsMTRR	KLOPRPYSCASSSLFHPGKLHFVFNIVEFLSTATTEVLRKGVCTGWLALL	496
CeMTRR	RLIPRPYSMS--YENRKARLIYSEMEFPATDGRHRSRKGATDVLNLS	497
HsCPR	RLQARYYSIASSSKVHPNSVHICAVVVEYETKAGR--INKGVATNWL---	494
	* * * * *	
	FAD	
HsMTRR	VASVLPONIHASHEDSGKALAPKISISPRTTNSFHLP-----DDPSIP	539
CeMTRR	R-----IGDKVOVLGKEPARFLPPLGMTKNSAGKLP	529
HsCPR	RAKE-----PVGNGGRALVPMFVRKSGFRLPFK-----ATTP	527
	. . . . .	
	NADPH	
HsMTRR	IIMVGPGTGIAPFIGFLQHREKLQECHPDGNFGAMW-LFFGCRHKDRDYL	588
CeMTRR	LLMVGPGTGVSFLSFLHFLRKLKODSPSDFVDVPRVLFVFGCRDSSVDI	579
HsCPR	VIMVGPGTGVAPFIGFIOERAWLROOGKE---VGETLLYYGCRSDDEDYL	574
	..*****. * . . . . *	
	NADPH	
HsMTRR	FRKELRHFLKHGILTHLKVSRDAPVGEELAPAKYVQDNIOHGGQVAR	638
CeMTRR	YMSELEMFVSEGILTDLIICESEQ-----KGERVQDGLRKYLDKVLP	621
HsCPR	YREELAQFHRDGLTQLNVAFSRE-----GSHKVYVCHLLKQDREHLWK	618
	. * * . . . . *	
	NADPH	
HsMTRR	ILLQE-NGHIYVCGDAKNMAKDVDHALVQIISKEVGVEKLEAMKTLATLK	687
CeMTRR	FLTASTESKIFICGDAKGMSKDVQCFSDIVASDQIGIPDLAKKLMOLK	671
HsCPR	LI--EGGAHIYVCGDARNMARDVONTFYDIAELGAMEHAQAVDYIKLM	666
	. . . . .	
	NADPH/FAD	
HsMTRR	EKRYLQDIWS	698
CeMTRR	KSDQYIEDVWG	682
HsCPR	TGGRYSLDVS	677
	* * . . .	

FIG. 4 page 2 of 2

FIG. 5A

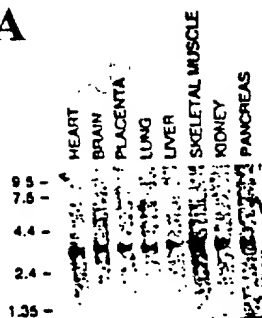


FIG. 5B





660750" 44E7260



FIG. 6

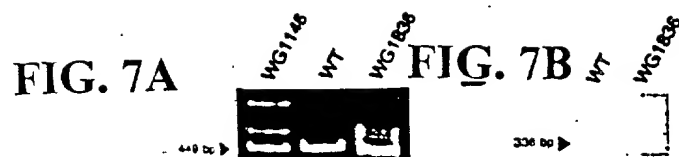


FIG. 7C

Position	Sequence	Accession/ Protein	Organism
572	CAPLLYCCNHPDMVLP	(AF025794) MTRR	[H. sapiens]
558	CEILLYYCCPSPDIDVLY	(AA0557) CPM	[H. sapiens]
559	CEILLYYCCPRAIDVLY	(D00101) CPM	[O. cuniculus]
560	CEILLYYCCPSPDIDVLY	(S71090) CPM	[D. melanogaster]
572	CVALLYCCNHPDMVLY	(P71116) CPM	[V. radiata]
573	CMVLYYCCNHPDIDVLY	(Z24418) CPM	[A. niger]
1281	CMVLYYCCPSPDIDVLY	(D14408) NOS I	[H. sapiens]
1009	CMVLYYCCPSPDIDVLY	(UC5810) NOS II	[H. sapiens]
1040	TPVLYYCCPSPDIDVLY	(L24314) NOS III	[H. sapiens]
380	CPVLYYCCPSPDIDVLY	(U85094) NOS	[O. cuniculus]
1009	CMVLYYCCPSPDIDVLY	(U44504) NOS	[G. gallus]
481	CMVLYYCCPSPDIDVLY	(M21004) SH	[E. coli]
915	CEVLYYCCPSPDIDVLY	(L26501) SH	[S. cerevisiae]
407	CMVLYYCCPSPDIDVLY	(Z23149) SH	[T. roseopersicina]
242	CLVLYYCCPSPDIDVLY	(X79417) FNR	[P. sativus]
251	CLVLYYCCPSPDIDVLY	(P00455) FNR	[S. glaucus]

FIG. 8A

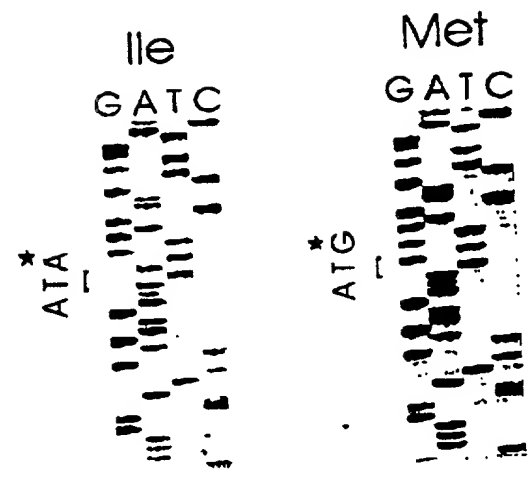
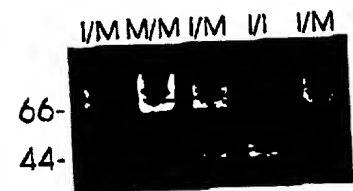


FIG. 8B



COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled HUMAN METHIONINE SYNTHASE REDUCTASE: CLONING, AND METHODS FOR EVALUATING RISK OF NEURAL TUBE DEFECTS, CARDIOVASCULAR DISEASE, AND CANCER, the specification of which

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as Application Serial No. \_\_\_\_\_  
and was amended on \_\_\_\_\_.

☐ was described and claimed in PCT International Application No. \_\_\_\_\_  
filed on \_\_\_\_\_ and as amended under PCT Article 19 on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Country	Serial Number	Filing Date	Priority Claimed?
			Yes/No
			Yes/No
			Yes/No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a) which became available between the filing date of the prior application and the national or PCT international filing date of this application:

# COMBINED DECLARATION AND POWER OF ATTORNEY

Serial Number	Filing Date	Status
09/232,028	January 15, 1999	Pending
60/071,622	January 16, 1998	Abandoned

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Paul T. Clark, Reg. No. 30,162, Karen L. Elbing, Ph.D. Reg. No. 35,238, Kristina Bieker-Brady, Ph.D. Reg. No. 39,109, Susan M. Michaud, Ph.D. Reg. No. 42,885.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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